UNIVERSITY OF TASMANIA

Novel short-chain quinones against mitochondrial dysfunction

Monila Nadikudi

MPharmSc, BPharm, GradCertRes

Pharmacy, School of Medicine, College of Health and Medicine

University of Tasmania

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*both authors have contributed equally

Statement of Co-authorship

Patent 1, Novel short-chain naphthoquinones against mitochondrial dysfunction:

Inventors: Gueven, N., Smith, J., Nadikudi, M., Woolley, K.L.

Located in Chapter 2 and 3

Gueven, N, Smith, J contributed to the conception and design of the research project and drafted significant parts of the patent

Nadikudi, M was the third author and along with Woolley, K.L contributed approximately 10 % to the planning, execution and preparation of the work for the patent.

Paper 1, Targeting mitochondrial function to treat optic neuropathy:

Authors: Gueven, N., Nadikudi, M., Daniel, A., & Chhetri, J.

Located in Chapter 1

Gueven, N contributed to the conception and design of the review paper and edited significant parts of the paper

Nadikudi, M was the second author and along with Daniel, A and Chhetri, J drafted significant parts of the paper

Gueven, N, Nadikudi, M, Daniel, A and Chhetri, J contributed approximately 40 %, 25 %, 25 % and 10 % respectively to the planning, execution and preparation of the work for the paper.

Paper 2, Developing naphthoquinone derivatives for the treatment of mitochondrial dysfunction:

Authors: Woolley, K.L*., Nadikudi, M*., Koupaei, M.N., Corban, M., McCartney, P., Bissember, A.C., Lewis, T.W., Gueven, N. and Smith, J.A.

*Both authors have contributed equally

Located in Chapter 2

Gueven, N., Smith, JA., Woolley, KL. and Nadikudi M. contributed to the conception and design of the research paper and drafted significant parts of the paper

Nadikudi, M. and Woolley, KL. have performed majority of the experiments (90 %) while Koupaei, M.N. and Corban, M. have also contributed to performing the experiments (10 %)

Nadikudi, M. and Woolley, KL. have analysed all data

Gueven, N., Smith, JA., Bissember, A.C. and Lewis, T.W. have contributed reagents and materials.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

A/Prof. Nuri Gueven

Supervisor

Pharmacy, School of Medicine

College of Health and Medicine

University of Tasmania

Date: 20/08/2018

Prof. Ben Canny

Head of School

School of Medicine

College of Health and Medicine

University of Tasmania

Date: 23/08/2018

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Table of Contents

List of Abbreviations

Acronym	Definition
Acetyl-CoA	Acetyl-coenzyme A
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANS	Ataxia neuropathy spectrum
ASK1	Apoptosis signal-regulated kinase-1
ATP	Adenosine triphosphate
AVV	Adeno-associated virus
BHB	β-hydroxy butyrate
BHB-dh	β-hydroxy butyrate dehydrogenase
BLP	Basal lipid peroxidation
BSA	Bovine serum albumin
C-I	Complex I
C-II	Complex II
C-III	Complex III
C-IV	Complex IV
C-V	Complex V
CFF	Cambridge farm facility
CMC	Carboxymethyl cellulose

CoQ_{10}	Coenzyme Q_{10}
Cox10	Protoheme IX farnesyltransferase
CytC	Cytochrome C
CYP450	Cytochrome P 450
d	Days
DCPIP	Dicholoro-phenolindo-phenol
DMEM	Dubecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOA	Dominant Optic Atrophy
Drp1	Dynamin-related-protein-1
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
ETC	Electron transport chain
ETF	Electron transport flavoprotein
ETFDH	Electron transport flavoprotein dehydrogenase
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide reduced
FBS	Fetal bovine serum
FH	Fumarase
FRAS	Friedrich's ataxia rating scale
FRDA	Friedrich's ataxia
G6P	Glucose-6-phosphate

GABA	γ-amino butyric acid
GCL	Ganglion cell layer
GPT	Glutamate pyruvate transaminase
GPx1	Glutathione peroxidase 1
GSH	Glutathione, L-γ-glutamyl-L-cysteinyl-glycine
GSR	Glutathione reductase
GSSG	Glutathione disulphide
H & E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney cells
HepG2	Human hepatic carcinoma cells
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
$HO^{\cdot}$	Hydroxyl free radical
IMM	Inner mitochondrial membrane
IF	Immunofluorescence
INL	Inner nuclear layer
IPL	Inner plexiform layer
IRS	Insulin receptor substrates
IS	Inner segment
KD	Ketogenic diet
α-KGDH	α-ketoglutarate dehydrogenase
KH_2PO_4	Potassium dihydrogen phosphate
LDH	Lactate dehydrogenase

LHON	Leber's hereditary optic neuropathy
LRB	Lactate reaction buffer
LS	Leigh syndrome
MAO	Monoamine oxidase
MB	Methylene blue
MAPK	Mitogen-activated protein casade
MELAS	Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy and ragged red fibres
MFN	Mitofusin
$MgCl_2$	Magnesium chloride
MIEF1	Mitochondrial elongation factor-1
MitoQ	Mitoquinone mesylate
MM	Mitochondrial mass
mPTP	Mitochondrial permeability transition pore
MS	Multiple sclerosis
MSP	Medical Science Precinct
mtDNA	Mitochondrial DNA
MTPD	Mitochondrial Trifunctional Protein Deficiency
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NARP	Neuropathy, ataxia and retinitis pigmentosa
nDNA	Nuclear DNA
NGFR	Nerve growth factor receptor
NQO1	NAD(P)H quinone oxidoreductase-1
O_2	Molecular oxygen
$O^{2\cdot-}$	Superoxide radical
OKR	Optokinetic response
OMR	Optomotor response
OMM	Outer mitochondrial membrane
ONL	Outer nuclear layer
OPA1	Optic atrophy-1
OPL	Outer plexiform layer
OS	Outer segment
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDCD	Pyruvate Dehydrogenase Complex Deficiency
PDH	Pyruvate dehydrogenase
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1-alpha
PGDFR	Platelet-derived growth factor receptor
PI3K	Phosphoinositide-3-kinase

PL	Photoreceptor layer
PMS	Phenazine methosulphate
POLG	Polymerase gamma
PPARγ	Peroxisome proliferator-activated receptor gamma
PPi	Inorganic phosphate
PPP	Pentose phosphate pathway
PTZ	Pentylenetetrazole
Q	Quinone
QH_2	Hydroquinone
Reagent A	Surfactant
Reagent B	Alkaline copper tartrate
Reagent S	Folin's agent
RGC	Retinal ganglion cells
RIR	Retinal ischemic reperfusion
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
Ru5P	Ribulose-5-phosphate
SAR	Structure-activity relationship
SD	Standard deviation
SDH	Succinate dehydrogenase
SIRT1	Silent information regulator protein-1
SMA	Spinal muscle atrophy
SOD	Superoxide dismutase

SS peptide	Szeto-Schiller peptide
TCA	Tricarboxylic acid cycle
TEM	Transmission electron microscopy
Tris HCl	Trizma hydrochloride
UTAS	University of Tasmania
UV	Ultraviolet
VDAC	Voltage-dependent anion channels
VK	Vitamin K
VKORC1	Vitamin K reductase C1
WST-1	Water-soluble tetrazolium-1

List of Figures

List of Tables

Abstract

Mitochondria regulate crucial cellular processes such as energy production in the form of adenosine triphosphate (ATP), Ca^{2+} homeostasis, cellular redox status and cell death. Consequently, cells and tissues that depend on these functions are susceptible to mitochondrial dysfunction. Any insult or genetic predisposition that leads to mitochondrial dysfunction can lead to a range of disorders that can manifest in many different tissues. Mitochondrial diseases, caused by mutations in mitochondrial DNA typically demonstrate severe neurological pathologies. These disorders are usually associated with defects in oxidative phosphorylation, reduced ATP levels, increased oxidative stress and decreased cellular viability. A class of compounds known as quinones are reported to protect against mitochondrial dysfunction. Currently, only one benzoquinone, idebenone, is approved in Europe to specifically treat a mitochondrial disease, while a few other drug candidates are in clinical development. Idebenone is reported to rescue cellular ATP levels and act as an antioxidant. Despite reported pre-clinical and clinical efficacy, no causative mode of action has been confirmed for this class of molecules that goes beyond pure associations. It is thought that quinones need to be bio-activated by cellular reductases such as NAD(P)H quinone oxidoreductase 1 (NQO1) for their therapeutic activity. Due to a rapid first pass metabolism and dependence on a single enzyme (NQO1) for its bio-activation, the clinical efficacy of idebenone is limited. Naphthoquinones, structural analogues of vitamin K, have also been investigated as potential therapeutic molecules to treat mitochondrial dysfunction-induced neurological disorders. In fact, growing evidence suggests that naphthoquinones could be beneficial to counteract mitochondrial dysfunction that is associated with neurological disorders such as epilepsy and Parkinson's disease.

In this study, I characterised more than 110 novel short-chain naphthoquinones (SCQs) to protect against mitochondrial dysfunction while working towards a better understanding of their mechanism of action. These SCQs were characterized for their ability to protect against mitochondrial dysfunction *in vitro*. Consistent with previous data from benzoquinones, most novel SCQs were mainly bioactivated by NQO1, while some were also bio-activated by other unidentified cellular reductases. Overall, more than 20 of our SCQs showed significantly better *in vitro* cytoprotection against the mitochondrial complex-I (C-I) inhibitor, rotenone, compared to idebenone. For the first time, together with my collaborators from Department of Chemistry, we established a clear structure-activity relationship and identified optimal chemical characteristics for naphthoquinone-based SCQs. This included a specific solubility range with a defined balance between side-chain polarity, fattiness and the presence of specific functional groups attached to the quinone core. Interestingly, more than half of the novel SCQs significantly increased basal ketone levels in cellular supernatant suggesting possible upregulation of fatty acid metabolism. In contrast to what is currently believed as the mechanism of action of quinones, a striking lack of correlation between cytoprotection by our SCQs and their effects on ATP levels, lipid peroxidation, lactate or ketone levels suggests that the mechanism of action of these compounds is likely much more complex than previously anticipated.

One of the most common, maternally-inherited mitochondrial disorders, Leber's Hereditary Optic Neuropathy (LHON) is caused by mutations in the mitochondrial DNA that encode subunits of mitochondrial C-I. These LHON mutations result in mitochondrial C-I deficiency, decreased ATP generation and increased oxidative stress which cause reduced retinal thickness and ultimately loss of retinal ganglion cells (RGC). LHON patients show a rapid and progressive loss of visual acuity. However, a rare possibility of

partial recovery of vision was reported for some patients, sometimes even years after disease onset. Currently, idebenone is the only drug approved in the Europe for the treatment of LHON. In order to translate my *in vitro* findings to a disease model, two of the most promising cytoprotective novel SCQs that also significantly rescued ATP levels *in vitro* in the presence of the C-I inhibitor, rotenone, were tested in an *in vivo* mouse model of LHON. Intraocular injection of rotenone in wild-type C57Bl/6 mice significantly ($p<0.05$) reduced both retinal RGC counts and the retinal thickness, which was associated with rapid loss of visual acuity. After 70 days of oral treatment with the novel SCQs, visual acuity was restored significantly ($p<0.05$) better than sham or idebenone-treated mice, with improvements evident from the first week of treatment. Both SCQs significantly ($p<0.05$) protected against RGC loss and reduced retinal thickness under conditions where idebenone showed no efficacy. In addition, no overt signs of SCQ-induced toxicity were observed in any of the animals. Overall, my *in vitro* and *in vivo* data suggest that these novel SCQs can be developed into superior, effective and safe drugs to treat disorders associated with mitochondrial dysfunction.

Chapter 1: Introduction

1.1 Mitochondria

Mitochondria are multifunctional organelles that were believed to be exclusively maternally inherited until a recent report highlighted that this might be incorrect (Shiyu et al, 2018). Mitochondria display a high degree of plasticity and interconnectivity with other organelles. Mitochondria are double-membraned organelles that are the site of many metabolic and biosynthetic processes within the cell. Apart from generating energy in the form of adenosine triphosphate (ATP), mitochondria are essential for many processes including redox signalling, production and modulation of reactive oxygen species (ROS) (Maurya et al. 2015, Zorov et al. 2014), maintenance of calcium homeostasis (Giorgio et al. 2017, Ichas et al. 1994), thermoregulation (Chouchani et al. 2016, Nedergaard et al. 2001) and regulating cell death (Davis and Williams 2012, Vakifahmetoglu-Norberg et al. 2017, Wojtczak and Zabłocki 2008). The number of mitochondria per cell varies anywhere between hundreds to thousands depending on the metabolic demand by the cell (Floros et al. 2018, Robin and Wong 1988).

Mitochondrial morphology varies according to the individual needs of its host tissue. For example, within hepatocytes, the shape of mitochondria varied from small grain-like to branched, thread-like structures (Collins et al. 2002). Proteomic analysis of rat mitochondria extracted from heart, skeletal muscle and liver tissues reveal that although qualitatively very similar, the protein abundance in mitochondria of different tissue was quite different (Forner et al. 2006). For example, mitochondrial electron transport chain (ETC) complex proteins were abundant in heart mitochondria, urea cycle enzymes were abundant in the liver whereas in the skeletal muscle, several phosphatases and kinases that regulate energy metabolism were abundant (Forner et al. 2006).

Mitochondria from different organs perform different functions, which are dictated by the tissue's metabolic demand under normal physiological conditions as well as in environments that exert different form of stress onto the cells and tissues (Burté et al. 2015). For example, in organs with a high-energy demand, such as the heart, the mitochondrial volume represents 23-32 % of the total myocellular volume of a cardiomyocyte (Lopaschuk et al. 2010, Murphy et al. 2016). These mitochondria in cardiomyocytes predominantly oxidise either free fatty acids bound to albumin or fatty acids released from triacylglycerol in chylomicrons or very-low-density lipoproteins, to generate 50-70 % of the required ATP to sustain cardiac function (Lopaschuk et al. 2010, Murphy et al. 2016). Glycolysis, lactate and ketone bodies are also used if additional energy is required (Lopaschuk et al. 2010, Murphy et al. 2016). In contrast to the heart, brain mitochondria use glucose as a primary source for energy generation under normal physiological conditions (Magistretti and Chatton 2005). Brain mitochondria use almost 31 mmol of glucose per 100 g of brain weight/min (Kety and Schmidt 1948). In the brain, apart from energy generation, glucose is also used as a substrate for the synthesis of building blocks of amino acids, proteins and neurotransmitters by the pentose phosphate pathway (Magistretti and Chatton 2005).

Within healthy cells, mitochondria are dynamic organelles that undergo continuous biogenesis, repair and degeneration by mitophagy to maintain cellular health and ensure metabolic homeostasis. The dynamic nature of mitochondrial morphology is regulated by either fusion or fission where two organelles merge or the one organelle separates into two or more successors respectively (Skulachev 2001). Mitochondrial fusion facilitates the exchange of metabolites between the intermembrane space and mitochondrial matrix. Stressors, such as nutrient deprivation, force mitochondria to rely on oxidative phosphorylation (OXPHOS). Such conditions stimulate fusion of functional mitochondria

with dysfunctional mitochondria that allows the sharing of components to keep up with cellular energy demands (Rossignol et al. 2004). Mitochondrial fission balances mitochondrial fusion by creating daughter mitochondria of appropriate size to transport them along the microtubule network. Hence, mitochondrial fusion and fission allow reorganisation and division to daughter mitochondria, of which the damaged ones can be eliminated by mitophagy. (Twig et al. 2008, Zimmermann and Reichert 2017). Overall, our current understanding of mitochondria depicts an extremely dynamic organelle that rapidly responds to cellular and environmental stimuli to maintain cellular metabolism and homeostasis.

1.1.1 Structure and organisation of mitochondria

Mitochondria are composed two phospholipid membranes that contain multiple integral and peripheral proteins separated by an inter membrane space. The outer mitochondrial membrane (OMM) separates the mitochondrion from the cytoplasm and the inner mitochondrial membrane (IMM), which is folded into cristae. The IMM encloses the mitochondrial matrix, which hosts ribosomes, mitochondrial DNA (mtDNA), various soluble enzymes, membrane-bound enzyme complexes of the electron transport chain that are responsible for generating energy, inorganic ions and metabolites (Figure 1) (Campbell and Reece 2005). The OMM contains voltage-dependent anion channels (VDAC) which regulate membrane permeability by allowing compounds of up to 5000 Da to pass across the membrane freely. Due to the impermeability of larger molecules through the OMM, enzyme composition of the intermembrane space significantly differs to that of the cytoplasm (Campbell and Reece 2005).

Cellular ATP production mainly takes place at the inner mitochondrial membrane at the mitochondrial matrix side, while the intermembrane space acts as a proton depository. The IMM contains the entire respiratory chain along with the ATP synthase complex and several specific metabolite transporters for ATP, adenosine diphosphate (ADP), inorganic phosphate and other respiratory substrates.

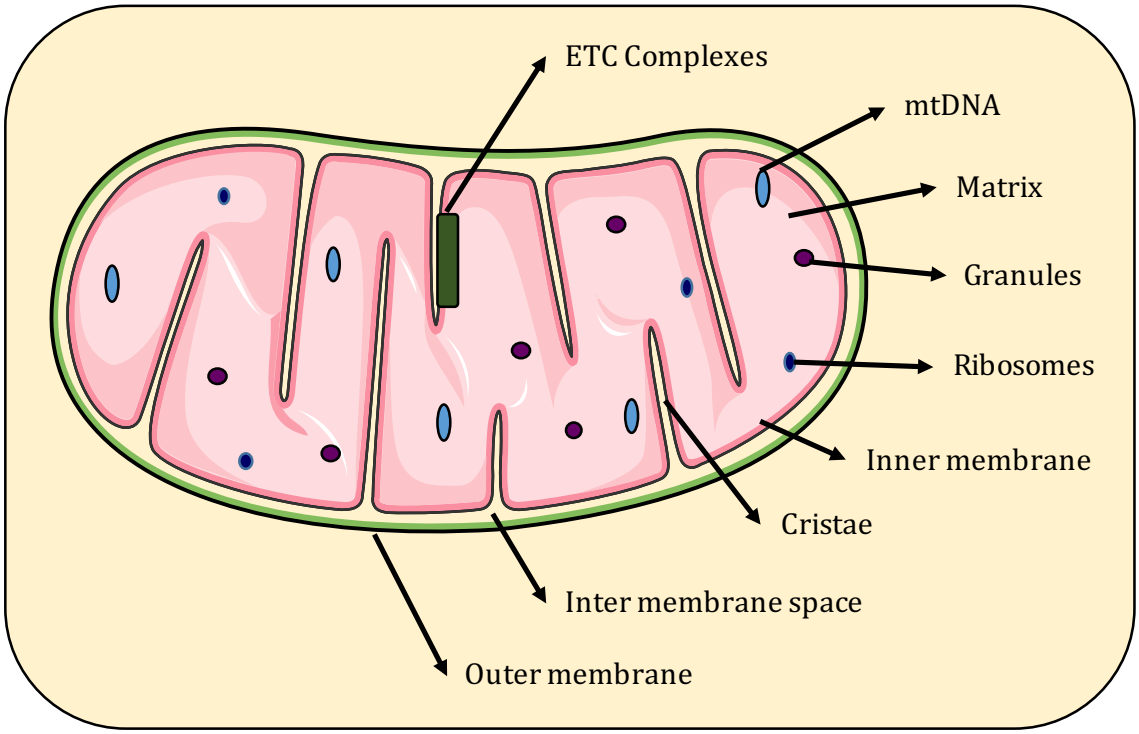


Figure 1: Schematic representation of mitochondrial structure. Mitochondria are organelles surrounded by two membranes. The inner mitochondrial membrane that is folded heavily into cristae hosts components of the mitochondrial electron transport chain (ETC). The matrix contains enzymes and co-enzymes involved in various energy generation pathways apart from electron mediators, mitochondrial deoxyribonucleic acid (mtDNA) and ribosomes.

The mitochondrial ETC complexes (C-I to C-IV) along with the ATP synthase complex (C-V) are localised inside the IMM (matrix side) and are specifically aggregated within the cristae as protein super complexes (Davies et al. 2011, Klotzsch et al. 2015, Wilkens et al. 2013). Each mammalian cell contains between hundreds to thousands of mtDNA molecules (Robin and Wong 1988, Satoh and Kuroiwa 1991, Sender et al. 2016). mtDNA are largely believed to be maternally inherited exclusively from the mother since paternal mtDNA was believed to be destroyed after fertilisation (Al Rawi et al. 2011, Politi et al. 2014). However, paternal leakage was occasionally observed in Drosophila (Kondo et al, 1992; Nunes et al, 2013) and mouse (Gyllensten et al, 1991). In a case report, a single male human with exercise intolerance and mitochondrial myopathy was reported to possess biparental mtDNA inheritance, ~90 % identical to the paternal haplotype in the skeletal muscle (Schwartz & Vissing, 2002). However, paternal inheritance was not seen in any other tissues (Schwartz & Vissing, 2002). In a recent study, biparental inheritance (24 % to 76 %) of mtDNA was observed in 17 members in 3 separate multigeneration families (Shiyu et al, 2018). Therefore, although mitochondria are largely maternally inherited, paternal transmission of mtDNA may also coexist.

Mammalian mitochondria are composed of over 1500 proteins, of which only 13 are encoded by the mtDNA, whereas the rest of them are nuclear encoded (Neupert and Herrmann 2007). The IMM is highly selective with regards to the permeability of metabolites and substrates, which is one of the prerequisites for energy production (Wojtczak and Zabłocki 2008). It is thought that its selectivity is also reflected by the unique structural composition of the IMM of about 80 % proteins and only 20 % phospholipids, compared to the outer mitochondrial membrane with a protein:lipid ratio of 1:1 (Giorgi et al. 2015, Wojtczak and Zabłocki 2008).

1.1.2 Energy generating pathways

All cells need energy to sustain their cellular functions. The central role of mitochondria is to generate chemical energy in the form of ATP. The energy status of the cell is sensed by multiple transcription factors, cofactors, hormones, kinases and nuclear receptors in the form of the adenosine monophosphate (AMP) to ATP ratio, the ratio of oxidised to reduced nicotinamide adenine dinucleotide (NAD^+:NADH) or the levels of acetyl coenzyme A (acetyl-CoA) (Nunnari and Suomalainen 2012). Increased energy demands can be met by increasing the activity of one of the energy generating pathways as well as increasing mitochondrial mass by upregulating mitochondrial biogenesis (Hoppeler and Flueck 2003). Cells generate the majority of their ATP by mitochondrial respiration, which metabolises fuel molecules such as sugars, fats and amino acids (Figure 2). However, the specific cell type, its physiological status and the availability of specific nutrients determine the type of fuel molecules and the pathway/s used to meet cellular ATP demand. For example, mitochondria from the heart predominantly use β-oxidation of fatty acids to produce ATP (Murphy et al. 2016), in contrast to brain mitochondria that predominantly use glucose-derived products (Mergenthaler et al. 2013). During fasting or under conditions of reduced glucose availability, neurons and glial cells can metabolise ketones for generating energy. Since the blood-brain barrier is impermeable to lipids, these are first catabolised in the liver to ketone bodies that are then transported to the brain to be used for energy generation (Morris 2005).

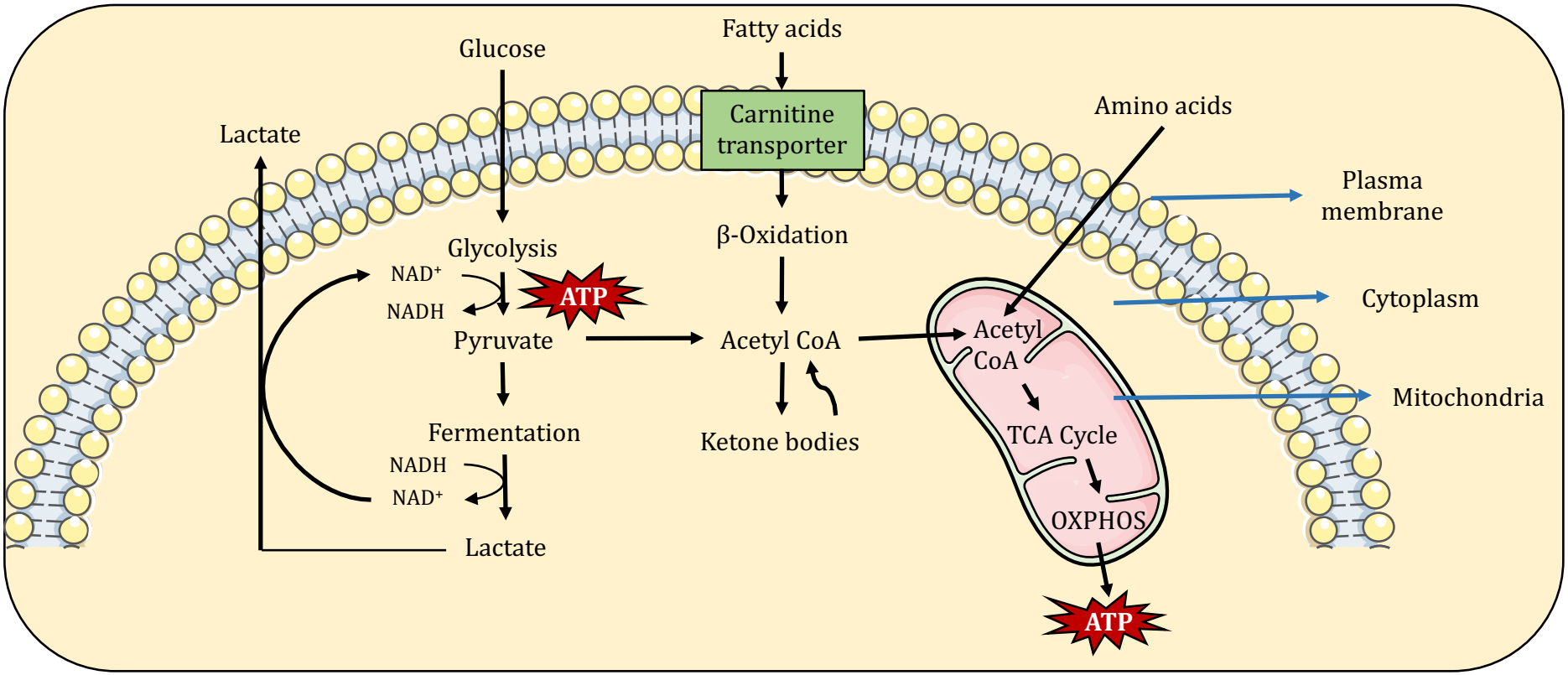


Figure 2: Energy generating pathways. Mitochondria can generate energy (adenosine triphosphate, ATP) from using sugars, fats or amino acids by glycolysis or β-oxidation pathways. The initial products of glycolysis and fatty acid oxidation can enter mitochondria by specialised transporters to undergo further reactions to generate acetyl coenzyme A (acetyl-CoA) that feeds into tricarboxylic acid (TCA) cycle and ultimately to the mitochondrial electron transport chain (ETC) generating more energy. The electron donors, like nicotinamide adenine dinucleotide (NAD^+) can be recycled by using the products of glycolysis in fermentation, resulting in the generation of lactate.
NADH, Nicotinamide adenine dinucleotide, reduced; OXPHOS, oxidative phosphorylation.

1.1.2.1 Glycolysis

Glycolysis is the metabolism of glucose to pyruvate by a series of enzyme-catalysed reactions that include the degradation of the glucose to pyruvate, phosphorylation of ADP to ATP along with the formation of NADH from NAD^+. Glycolysis, in the presence of oxygen, exhibits a high ratio of ATP:ADP and NADH/NAD^+ (Hume and Weidemann 1979, Wojtczak and Zabłocki 2008). In the first step of glycolysis, glucose is broken down to glucose-6-phosphate (G6P) (Figure 3). Depending on the current needs of the cell and its cytosolic nicotinamide adenine dinucleotide phosphate ($NADP^+$) concentrations, G6P either enters glycolysis or the pentose phosphate pathway (Vander Heiden et al. 2001).

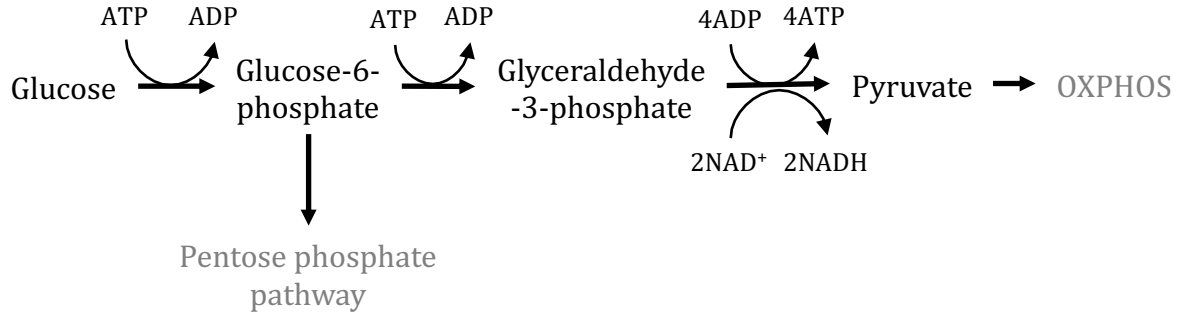


Figure 3: Glycolysis. Glucose is metabolised by glycolysis to glucose-6-phosphate (G6P) at the expense of one molecule of adenosine tri-phosphate (ATP). G6P is further metabolised to pyruvate, ultimately resulting in a gain of two molecules of each ATP and nicotinamide adenine dinucleotide (NAD^+) overall. Pyruvate, the end-product of glycolysis can enter the mitochondria to be metabolised in by the tricarboxylic acid (TCA) cycle.
ADP, Adenosine diphosphate; NADH, Nicotinamide adenine dinucleotide, reduced.

Although glycolysis is a far less efficient way to generate energy (two ATP for every molecule of glucose) when compared to OXPHOS (up to 36 ATP), proliferating cells in culture with a constant supply of glucose nearly exclusively use glycolysis not only to

generate ATP, but to also generate glycolytic intermediates that are shuttled and used to support anabolic reactions in cells and for the synthesis of building blocks of cell proliferation (DeBerardinis et al., 2008; Lunt and Vander 2011). The G6P can enter the pentose phosphate pathway (PPP) to produce DNA, RNA and electron donors NADH, FADH2 (Patra and Hay, 2014). Other glycolytic intermediate, 3-phosphoglycerate can be used for the synthesis of nucleotides and proteins via serine synthesis pathway (Yang and Vousden, 2016; DeBerardinis et al., 2008).

The glycolytic pathway of energy generation is preferred by the cultured cells in conditions where only about 5 % or the entire pyruvate is used by mitochondria for OXPHOS (Nelson et al. 2008). Apart from that, cells can also generate energy during hypoxic conditions by anaerobic glycolysis (Nelson et al. 2008). Importantly, given that most cells used for *in vitro* cell culture experiments are of cancerous origin, it is necessary to point out that regardless of oxygen availability, cancer cells predominantly use glycolysis for ATP production, which results in the production of significant amounts of lactate. This process is known as aerobic glycolysis or Warburg effect (Potter et al. 2016, Vander et al. 2009, Warburg et al. 1927).

The end-product of glycolysis, pyruvate, still contains the majority of the potential chemical energy of glucose and can therefore be used in subsequent reactions. In the presence of oxygen, pyruvate is converted to acetyl CoA before being fully metabolised in the mitochondrial OXPHOS. In contrast, in the absence of oxygen, pyruvate is converted to lactate to recycle NAD+ from NADH, which is essential to sustain the initial steps of glycolysis (Vander et al. 2009). For cells solely relying on glycolysis as a source of energy, protons released from the breakdown of ATP accumulate within these cells since they cannot be buffered by glycolytic cells. This resultant proton accumulation leads to a

decrease in cellular as well as blood pH causing acidosis (Fall and Szerlip 2005). Increased glycolysis is accompanied by the release of lactate into the systemic circulation which can, in severe cases, lead to the clinical symptom of lactic acidosis (Robergs et al. 2004).

1.1.2.2 Pentose phosphate pathway

In animal cells, glucose is broken down to G6P during the first step in glycolysis before being metabolised to pyruvate (Nelson et al. 2008). However, depending on the cellular metabolic demands and environmental factors, G6P can enter into the pentose phosphate pathway (PPP), also known as phospho-gluconate pathway or hexose monophosphate shunt. All PPP enzymes are located in the cytosol where they generate the nucleotide-precursor, ribose-5-phosphate that is required to synthesise RNA and DNA (Kruger and von Schaewen 2003). The PPP is a major source of NADPH generation and is also involved in the production of precursors for the synthesis of aromatic amino acids. Therefore, not surprisingly, the PPP is mainly active in cells and tissues that are rapidly proliferating and/or are associated with higher levels of oxidative stress (Du et al. 2013). In the presence of oxidative stress, NADPH generated by PPP is used to regenerate the endogenous antioxidant, glutathione from the oxidised glutathione disulphide (GSSG) to the reduced glutathione (GSH) (Nelson et al. 2008) (See section 1.1.4 and Figure 11).

NADPH, generated by the PPP is also required by tissues that synthesise fatty acids, like the liver and adipose gland as well as tissues involved in cholesterol and steroid hormone synthesis like the adrenal gland and gonads (Patra and Hay 2014, Raïs et al. 1999). The

PPP is composed of two phases, the oxidative and non-oxidative phase. In the first step of the PPP, G6P is oxidised to ribulose-5-phosphate (Ru5P) while two molecules of $NADP^+$ are reduced to NADPH (Figure 4). The resultant Ru5P can either be converted to ribose-5-phosphate as a precursor for nucleotides, coenzymes, DNA and RNA synthesis or it can be recycled to G6P in the second, non-oxidative phase of the PPP (Horecker 2002, Nelson et al. 2008, Riganti et al. 2012).

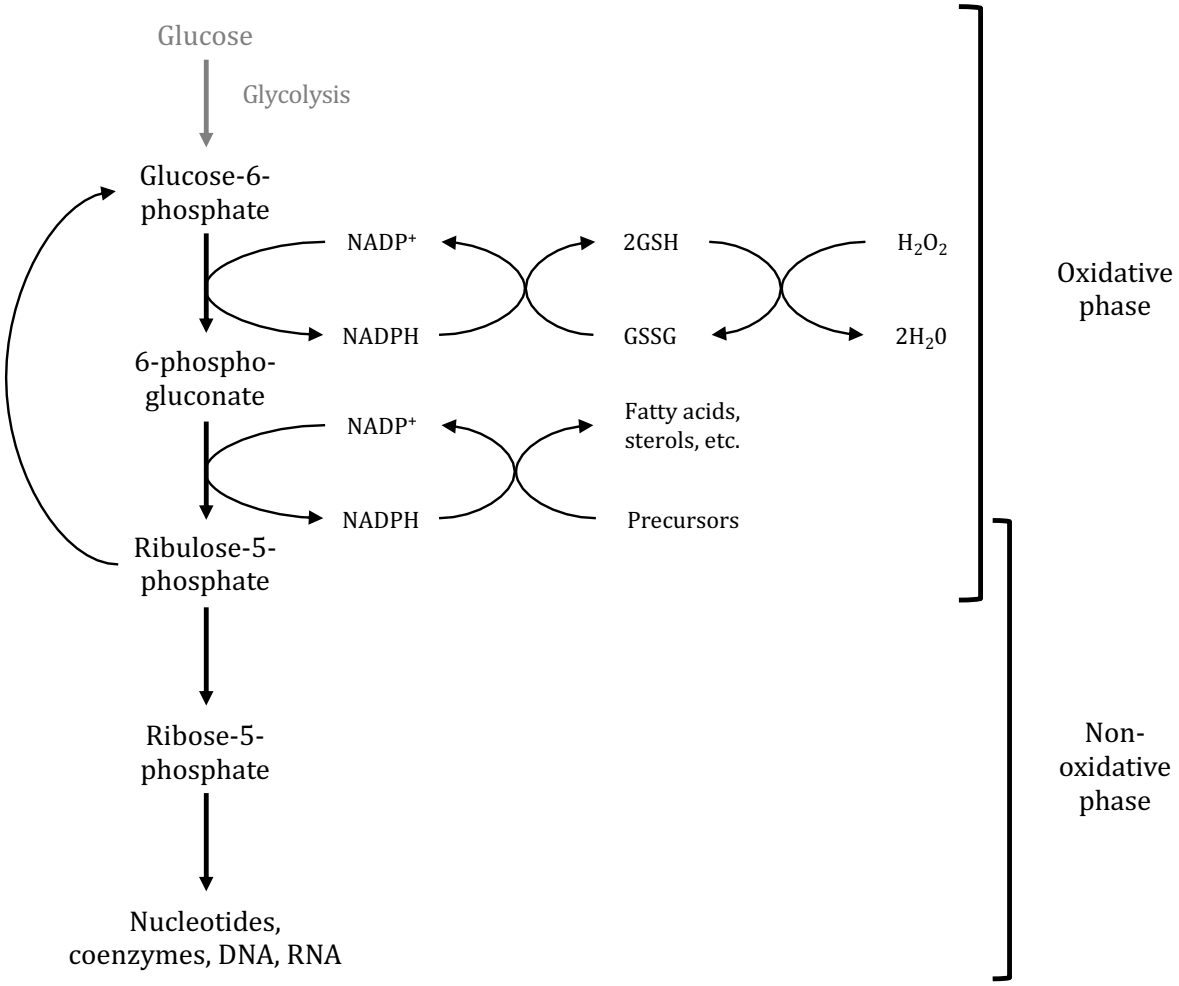


Figure 4: Pentose phosphate pathway. The pentose phosphate pathway (PPP) uses the intermediate of glycolysis, glucose-6-phosphate to generate ribulose-5-phosphate (Ru5P) while reducing $NADP^+$ to NADPH. This reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) can be used to convert oxidised glutathione (GSSG) to reduced glutathione (GSH) which can detoxify oxidative radical species. NADPH is also used to synthesise fatty acids. Ru5P can be further metabolised into ribose-5-phosphate that is used for the synthesis of nucleotides for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

1.1.2.3 β-oxidation of fatty acids

In addition to glucose, fatty acids are an important, abundant source of cellular energy. Under physiological conditions tissues under physiological conditions use sugar and fatty acids at different rates for mitochondrial ATP production. However, it is possible to adapt the body to near exclusive use of fatty acid-dependent respiration by dietary modification (Rho and Stafstrom 2012). Fatty acids are oxidised to acetyl-CoA by β-oxidation and the resultant acetyl-CoA feeds into the TCA cycle for further oxidation by OXPHOS to produce chemical energy (Figure 5) (Nelson et al. 2008). Cells obtain fatty acids either from the diet, from cellular storage vesicles or from fat that is synthesised *de-novo* in tissues such as the liver. The enzymes for β-oxidation of fatty acids are located in the mitochondrial matrix as well as in the liver peroxisomes (Schulz 1991).

Fatty acids containing 12 or less carbons can easily enter the mitochondria without any transporters. However, most of the dietary fats as well as the ones that originate from adipose tissue are composed of 14 or more carbons. These fatty acids require a special transport system known as the carnitine shuttle to transport them into mitochondria for metabolic degradation (Kerner and Hoppel 2000, Schulz 1991). This mitochondrial shuttle occurs in three steps. In the first step, fatty acids are converted to fatty acyl-CoA, at the expense of one ATP molecule, catalysed by the enzyme acyl-CoA synthetase (thiokinase) located on the OMM. Subsequently, fatty-acyl CoA is transiently attached to the hydroxyl group of carnitine located on the OMM forming fatty-acyl-carnitine catalysed by carnitine acyl transferase-1 before forming a carnitine ester at the outer surface of the IMM. This fatty acyl carnitine ester then enters the mitochondrial matrix via the carnitine transporter located on the IMM. In the final step of the carnitine shuttle,

with the help of coenzymes located on the IMM, fatty-acyl-CoA is regenerated and free carnitine is released (Figure 5) (Kerner and Hoppel 2000, Schulz 1991).

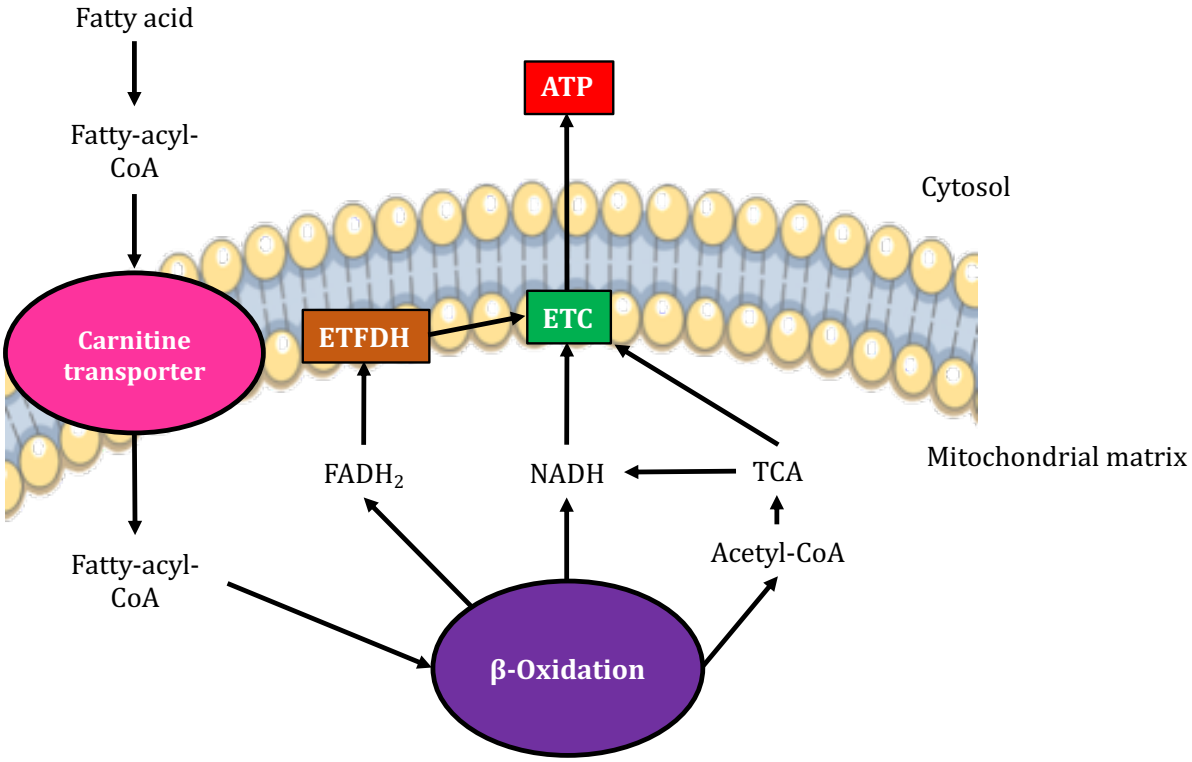


Figure 5: Mitochondrial β-oxidation of fatty acids. Fatty acids, converted to fatty acyl coenzyme A (fatty-acyl-CoA) enter mitochondria with the help of the carnitine transporter. Once inside the mitochondrial matrix, fatty-acyl-CoA is oxidised by β-oxidation resulting in the generation of the acetyl coenzyme A (Acetyl-CoA) and reduced electron carriers' nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). Acetyl-CoA enters the tricarboxylic acid cycle (TCA), NADH feeds electrons into complex-I of the mitochondrial electron transport chain (ETC), whereas $FADH_2$ feeds to complex III of ETC via electron transport flavoprotein dehydrogenase (ETFDH) ultimately resulting in the generation of energy (adenosine triphosphate, ATP).

This carnitine mediated fatty acid entry process is the rate-limiting step of β-oxidation of fatty acids. Once in the matrix, fatty-acyl-CoA undergoes multiple repeats of a four-step dehydrogenation process called β-oxidation, generating acetyl-CoA, NADH and reduced flavin adenine dinucleotide ($FADH_2$) until the entire fatty acyl-CoA is broken down to acetyl-CoA (Figure 5) (Longo et al. 2006, Stephens et al. 2013).

The generated acetyl–CoA enters the TCA cycle whereas the electron carriers donate the electrons to mitochondrial ETC. While NADH feeds electrons to C-I of ETC, the reduced flavoprotein, $FADH_2$ is re-oxidised by electron transport flavoprotein (ETF) and subsequently transferred to ETF dehydrogenase (ETFDH) embedded in the IMM (Figure 5). ETFDH is a flavoprotein that feeds electrons to C-III of the mitochondrial ETC via the electron carrier coenzyme Q_{10} (CoQ_{10}) (Gregersen et al. 2008, Houten and Wanders 2010).

In the liver, β-oxidation of fatty acids also occurs in the peroxisomes, where very long chain fatty acids are broken down to medium chain fatty acids that are further metabolised in the mitochondria to ketone bodies which are used as energy-rich molecules (Lazarow 1978). The process of peroxisomal degradation of fatty acids is very similar to the mitochondrial pathway. In the first step of converting fatty acids to acyl-CoA in the peroxisomes, an electron transfer occurs from acyl-CoA via $FADH_2$ to molecular oxygen, which generates hydrogen peroxide (H_2O_2) with the potential to cause oxidative damage. To prevent this liability, peroxisomes contain the endogenous antioxidant catalase that cleaves the generated H_2O_2 to molecular oxygen and water and CoQ_{10} that prevents lipid peroxidation due to the oxidative stress (Schulz 1991).

Overall, each cycle of β-oxidation yields an acetyl-CoA, one NADH and two $FADH_2$ molecules as energy rich electron molecules. Acetyl-CoA is further oxidised by entering into TCA cycle to generate one ATP, three NADH and two $FADH_2$ molecules. For each molecule of NADH and $FADH_2$ donated to the mitochondrial ETC, 2.5 and 1.5 molecules of ATP are generated respectively. Therefore, for each acetyl CoA oxidised, 11.5 molecules of ATP are generated (Longo et al. 2006, Nelson et al. 2008, Houten and Wanders 2010).

1.1.2.4 Tricarboxylic acid cycle

Energy-rich molecules including glucose, fats and some amino acids are oxidised to acetyl-CoA as a first step of cellular respiration prior to feeding acetyl-CoA into the tricarboxylic acid (TCA) cycle (also known as citric acid cycle or Krebs cycle) (Briere et al. 2006).

As the first step, pyruvate, derived from sugars via glycolysis is oxidised to acetyl-CoA in an irreversible reaction known as oxidative carboxylation by the enzyme pyruvate dehydrogenase (PDH) located in the mitochondria. During this reaction, NAD^+ is reduced to NADH, which in turn feeds electrons into C-I of the mitochondrial ETC. The generated acetyl-CoA is oxidised in the TCA cycle by a series of reactions (Figure 6). While only one molecule of ATP is directly generated in the TCA cycle, during the conversion of succinyl CoA to succinate, four energy-rich electron carriers are generated in the form of three molecules of NADH and one molecule of $FADH_2$, which are fed into the ETC to use this energy for ATP production. For each molecule of NADH and $FADH_2$ fed into the ETC, 2.5 and 1.5 molecules of ATP are generated (Nelson et al. 2008).

Apart from generating energy-rich electron carriers, the metabolites of the TCA cycle are also important biosynthetic intermediates. For example, citrate is used in fatty acid biosynthesis (Bhaduri and Srere 1963, Kuhajda 2000), α-ketoglutarate and oxaloacetate are precursors for amino acid synthesis (Krappmann and Braus 2002, Xu et al. 2018), while oxaloacetate is also be used for gluconeogenesis (Sepa-Kishi et al. 2018, Yoon et al. 2001). Succinyl-CoA is a key intermediate for synthesising the porphyrin ring of haemoglobin, myoglobin and is also used to produce electron carriers like cytochrome (Nelson et al. 2008, Vakifahmetoglu-Norberg et al. 2017).

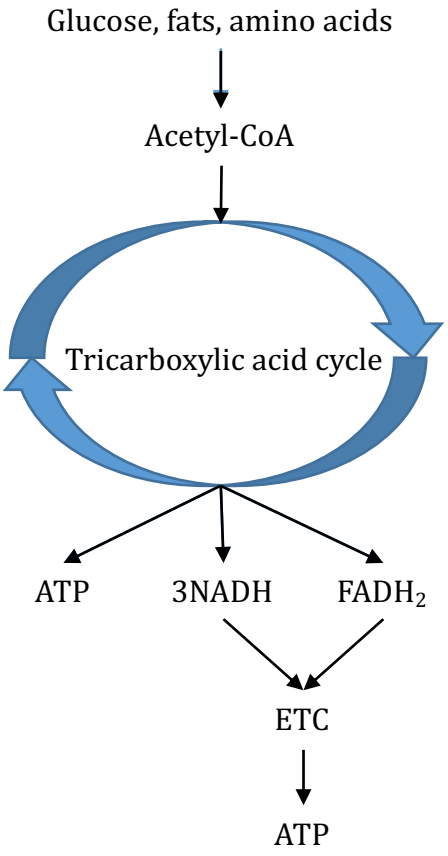


Figure 6: Tricarboxylic acid cycle. Energy rich substrates such as glucose, fatty acids and amino acids are broken down to acetyl coenzyme A (acetyl-CoA) that is subsequently fed into the tricarboxylic acid (TCA) cycle. This set of subsequent reactions directly generates one molecule of adenosine triphosphate (ATP) for every acetyl-CoA. TCA cycle also generates the reduced electron carriers, three molecules of nicotinamide adenine dinucleotide phosphate (NADH) and one molecule of flavin adenine dinucleotide ($FADH_2$) that are oxidised by the mitochondrial electron transport chain (ETC) to generate nine additional molecules of ATP.

In the absence of OXPHOS in the mitochondrial matrix, succinyl-CoA ligase, an enzyme of the TCA cycle, produces high energy phosphates via substrate-level phosphorylation (Johnson et al., 1998). During respiration inhibition due to ETC dysfunction in mouse liver, mitochondrial diaphorases contribute to regeneration of majority of the NAD+ pool (Kiss et al., 2014). This NAD+ pool in-turn supports the function of α-ketoglutarate dehydrogenase complex (KGDHC) in providing succinyl-CoA to succinyl-CoA ligase in the

TCA cycle, therefore maintaining substrate-level phosphorylation and generating ATP (Chinopoulos et al., 2010; Kiss et al., 2013). Therefore, even in the absence of OXPHOS, substrate-level phosphorylation generates ATP that is expelled into the cytosol therefore preventing ATP consumption by the mitochondria and hence avoiding cellular bioenergetic catastrophe (Chinopoulos et al., 2010; Chinopoulos, 2011).

1.1.2.5 Oxidative phosphorylation

Mitochondrial OXPHOS utilises the products of glycolysis and the TCA cycle to satisfy about 95 % of cellular ATP requirements *in vivo*. Energy-rich electrons derived from the breakdown of organic starting materials are sent via the mitochondrial ETC over multiple complexes and protein subunits to finally react with molecular oxygen (Campbell and Reece 2005). The process of the transferring electrons from one molecule to another is known as a redox reaction. The resultant energy from these reactions is used in the mitochondrial ETC to transport protons from the mitochondrial matrix, through the IMM to the intermembrane space. This process generates an electrochemical gradient across the IMM that is subsequently used by the mitochondrial ATP synthase (complex V) to synthesise ATP (Wojtczak and Zabłocki 2008).

The electron carriers of the mitochondrial ETC are composed of more than 85 proteins assembled into four membrane-embedded supramolecular complexes (Frey and Mannella 2000). Diffusible electron acceptors and donors connect these complexes, located in the cristae of the IMM. Briefly, electrons from NADH (C-I) and succinate (C-II)

are used to reduce CoQ_{10}. The reduced CoQ_{10} transfers electrons to cytochrome C (Cyt C) via C-III. Finally, C-IV completes the sequence of reactions by transferring electrons to molecular oxygen (Nelson et al. 2008, Wojtczak and Zabłocki 2008).

The largest complex of the ETC, C-I (NADH-ubiquinone oxidoreductase or NADH dehydrogenase), is composed of 43 proteins whose subunits are encoded by both nuclear and mitochondrial genes (Frey and Mannella 2000). NADH is oxidised by C-I by delivering its electrons to the flavin mononucleotide (FMN) site on C-1 (Figure 7). The resultant electrons from this reaction are then transported via iron-sulphur (Fe-S) clusters to the electron carrier CoQ_{10}, hence reducing it to the quinol (QH_2) form in the process. This reaction is coupled to the pumping four protons from matrix to the intermembrane space by separate subunits of C-I (Brandt et al. 2003, Ohnishi and Salerno 2005). Inhibition of C-I by drugs or mutations in essential C-I subunits typically hinders the flow of electrons to ubiquinone, which leads to the accumulation of NADH and an increase in ROS by electrons that leak from C-I onto oxygen (Figure 7). (Wojtczak and Zabłocki 2008). Ubiquinone, an electron carrier accepts electrons from both C-I and succinate dehydrogenase, which is an essential part of C-II. The only complex which is encoded entirely by nuclear DNA, C-II, is composed of four subunits, one containing flavin adenine dinucleotide (FAD) which is required for the oxidation of succinate to fumarate. In addition, a membrane subunit, containing three Fe-S clusters, aide in the transport of electrons to the next subunits. The electrons from the reduced ubiquinol are transported C-III (ubiquinol-cytochrome C oxidoreductase), where it is re-oxidised to Q, ready to diffuse back to C-I. C-III reduces ubiquinol to Q by a two-step process that involves the highly reactive intermediate semiquinone radical, which is associated with increased ROS formation. Four protons are released into the intermembrane space during the re-oxidation of ubiquinone (Wojtczak and Zabłocki 2008). In the last step of ETC, C-IV (Cyt

C oxidase) catalyses irreversible transfer of electrons from reduced Cyt C to oxygen resulting in the formation of water (Wojtczak et al. 2008). To reduce one molecule of oxygen, four molecules of reduced Cyt C and four protons are required. This last step of ETC adds four protons to the intermembrane space adding to the existing proton gradient. The resultant proton-motive force drives the conformational changes that allow ADP to react with inorganic phosphate to generate ATP by the enzyme ATP synthase (ATPase), which is sometimes also referred to as C-V (Figure 7) (Wojtczak and Zabłocki 2008).

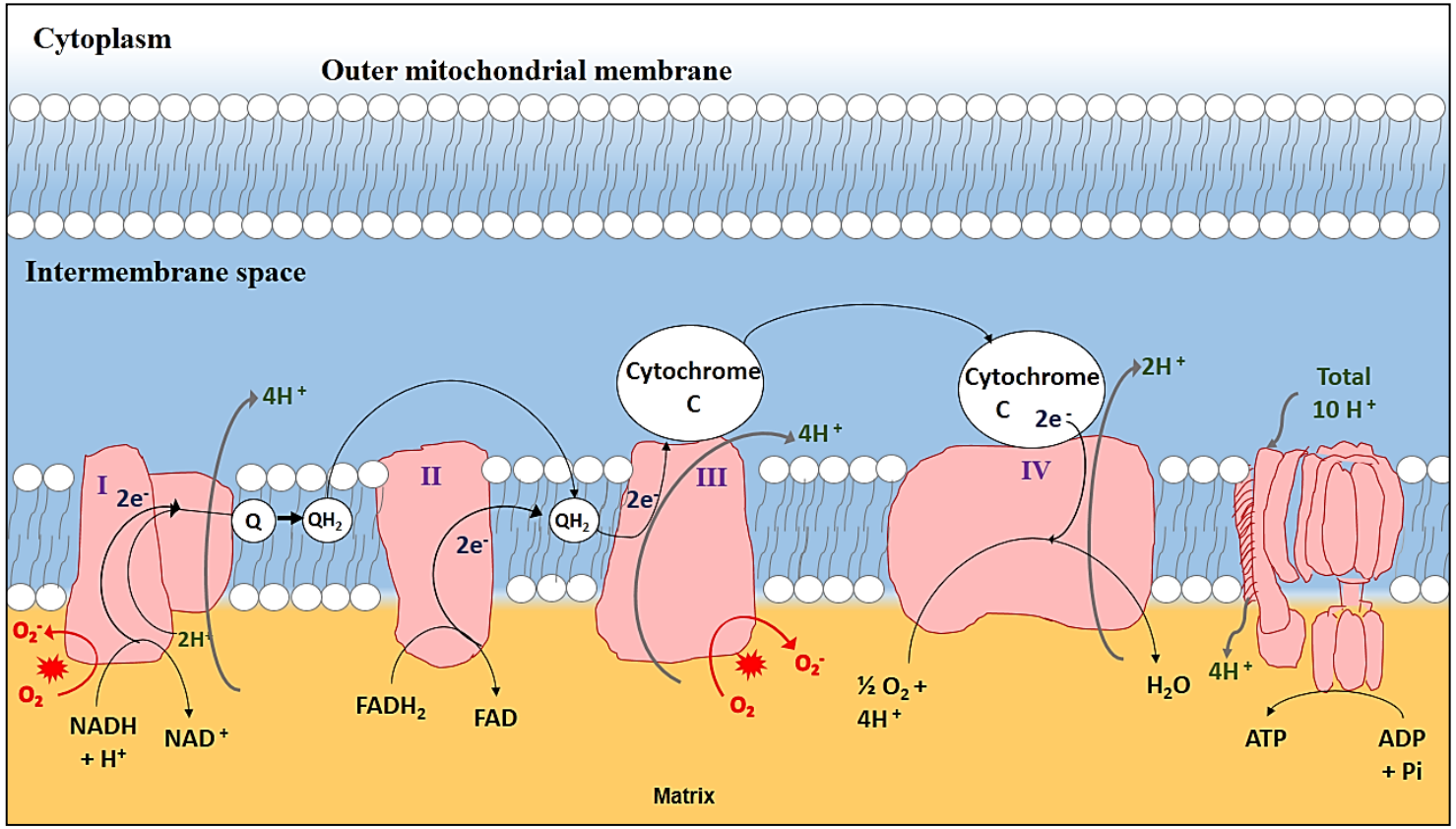


Figure 7: Schematic diagram of the mitochondrial electron transport chain. As a first step of oxidative phosphorylation (OXPHOS), a proton gradient is generated across the IMM, which drives the synthesis of adenosine triphosphate (ATP). Reduced electron carriers like nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) donate electrons (e^-) to the complex I and complex II of the ETC respectively. These electrons are subsequently transferred to ubiquinone (Q) thereby reducing it to ubiquinol (QH_2). The electrons are then transferred to complex III via cytochrome C and then to molecular oxygen (O_2) via complex IV to form water (H_2O). Following these reactions, the protons (H^+) that were transferred to the intermembrane space are shuttled back to the mitochondrial matrix by complex V (ATP synthase) by making conformational changes to F_1F_0 ATP synthase that results in the production of ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi).

1.1.2.6 Anaerobic energy generation

Under conditions of hypoxia or under conditions of dysfunctional mitochondria, NADH cannot be re-oxidised to NAD^+, which is required to sustain further glycolysis for ATP generation (Schurr 2006). Under these conditions, the product of glycolysis, pyruvate is reduced to lactate. During this process no ATP is synthesised, but instead NAD+ is regenerated from NADH, which is required for the initial steps of glycolysis and thereby supports glycolytic ATP generation (Figure 8) (Ido et al. 2011). Lactate was once considered as the toxic, waste product of glycolysis. However, lately lactate production has been recognised to not only regenerate NAD+ but also as a regulator of cellular redox status (Gao et al. 2018, Ido et al. 2011).

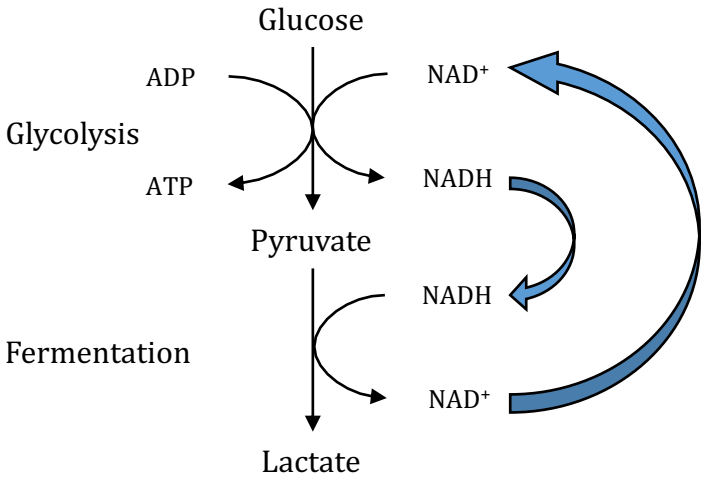


Figure 8: Lactate formation. Lactate is generated from the product of glycolysis, pyruvate. This process uses electrons from oxidising the reduced nicotinamide adenine dinucleotide (NADH) generated during glycolysis to recycle nicotinamide adenine dinucleotide (NAD^+) that is required to sustain the process of glycolysis.
ADP, adenosine diphosphate; ATP, adenosine triphosphate

During anaerobic physical exercise, sympathetic stimulation of muscle glycogenolysis results in lactate generation to keep up with the high ATP demand, which results in high lactate flux (Brooks 2009). The accumulated lactate can act as a gluconeogenic precursor during sustained exercise, by regenerating glucose from lactate in the liver at an expense of six molecules of ATP by a process known as Cori cycle (Jorge et al. 2017, Waterhouse and Keilson 1969). Lactate consumed by distal tissues or organs also affects the pH balance of the cells (Brooks 2009). Recent evidence supports the use of lactate as an alternative oxidative substrate for neurons along with ketone bodies (Pellerin and Magistretti 2012, Voutsinos-Porche et al. 2003). In fact, some studies report a preference of lactate over glucose as an oxidative substrate for neurons (Bouzier-Sore et al. 2006, Itoh et al. 2003, Ivanov et al. 2011) and glial cells (Drulis-Fajdasz et al. 2018). In astrocytes, lactate derived from glycogen in the astrocytes is also known to support the neuronal metabolism and neuronal plasticity by directly increasing ATP synthesis and/or by increasing the availability of NADH (Béland-Millar and Messier 2018). Although the use of lactate as energy source for brain metabolism is generally agreed on, it remains unclear whether lactate undergoes direct metabolism in the astrocytes or indirect metabolism by gluconeogenesis.

Overall, the fundamental roles of extra mitochondrial and mitochondrial energy generation involve different pathways. The choice of individual cells to generate energy by a particular mechanism depends on availability and type of nutrients, environmental factors as well as mitochondrial dynamics. Other prominent mitochondrial functions include the regulation of cellular redox status and the role of mitochondria in programmed cell death, which are discussed in the following sections.

1.1.3 Mitochondrial redox environment

The production of ROS by mitochondria was first described in 1966 (Jensen 1966). Most of the ROS generated endogenously by mitochondria under physiological conditions are the by-product of OXPHOS. However, due to their potentially damaging nature, ROS production is highly regulated by the mitochondria under normal physiological conditions (Turrens 2003). Only about 2 % of the total oxygen consumed by mitochondria is converted to ROS under normal physiological conditions, which is in contrast to levels as high as 11 % under pathological conditions (Aon et al. 2012, Zorov et al. 2014).

Most electrons that enter the mitochondrial ETC are used to reduce molecular oxygen to water. However, some electrons leak from the redox sites in the ETC, predominantly in C-I and C-III, which results in the one-electron reduction of molecular oxygen that generates superoxide ($O^{2\bullet-}$) (Adam-Vizi and Chinopoulos 2006). Due to the high reactivity of $O^{2\bullet-}$, H_2O_2 is generated in the presence of the enzyme superoxide dismutase (SOD). The resultant H_2O_2 reacts with metal cations in a Fenton reaction that produces the extremely reactive hydroxyl radical ($HO^{\bullet}$). Superoxide anion, hydroxyl, alkoxyl, peroxyl radicals along with hydrogen peroxide generated within the cell together constitute the majority of ROS.

The main sites of ROS generation within mitochondria are C-I and C-III (Figure 9) (Zorov et al. 2014). However, practically all other major mitochondrial protein complexes also have the potential to add to the production of ROS. Recent studies also demonstrated the involvement of C-II as well, but to a lesser degree (Korge et al. 2017, Quinlan et al. 2012). C-I and C-III inhibitors, like rotenone and antimycin respectively further exacerbate the

production of ROS (Chen et al. 2003). For example, C-I inhibition by rotenone enhances the reduction of the NADH reductase site, which increases the electron leak from C-I and results in the generation of ROS towards the mitochondrial matrix side (Li et al. 2003). Supporting the involvement of C-IV and C-V in ROS generation, addition of specific inhibitors KCN and oligomycin blocked ROS generation (Reichart et al., 2018; Ricci et al., 2003; Yoon et al., 2005).

Another mitochondrial enzyme, dihydroorotate (DHO) dehydrogenase (DHOdh), located in the IMM is also reported to generate ROS during the oxidation of DHO (Evans and Hedeel, 2004). This is supported by reduced ROS generation and apoptosis by downregulating or chemically inhibiting DHOdh in cancer cell lines (Hail et al., 2010). Other mitochondrial enzymes that are responsible for ROS generation include monoamine oxidase (MAO), α-ketoglutarate dehydrogenase (α-KGDH) and pyruvate dehydrogenase (PDH) (DiLisa et al., 2009). Apart from these enzymes, the senescence-related protein p66shc upon interaction with CytC serves as another site for mitochondrial ROS generation (Giorgio et al., 2005).

Mitochondrial ROS are either detoxified by antioxidant defence mechanisms within the mitochondria or at higher concentrations have the potential to cause mitochondrial damage (Chen et al. 2003). Mitochondrial ROS production is also induced by other factors like ubiquinone pools that can undergo incomplete, one-electron reduction resulting in the generation of semiquinone that can cause further cell damage (Murphy 2009). This was further confirmed by the fact that prevention of semiquinones also suppressed superoxide formation. For example, supplementing succinate as an electron donor to rodent heart mitochondria in the presence of C-III inhibitor, antimycin, prevented the formation of $O^{2\cdot -}$ (Rigoulet et al. 2011, Turrens et al. 1985).

ROS are generated outside the mitochondria under conditions where H_2O_2 enters the cytosol. There, it generates $HO^{\cdot}$ in the presence of transition metal cations via Fenton chemistry as follows (Wojtczak and Zabłocki 2008):

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^{-} + Fe^{3+}$$

Studies also reported the efflux of H_2O_2 from mitochondria to the cytosol under conditions of a high NADH/NAD^+ ratio in the mitochondrial matrix. This effect also occurs in the presence of high levels of ubiquinone electron transport coupled with high membrane potential and lack of ATP synthesis in the mitochondrial matrix (Angelova and Abramov 2016, Murphy 2009). Several other mitochondrial enzymes such as cytochrome P450 enzymes (CYP450), monoamine oxidase (MAO), α-ketoglutarate dehydrogenase (α-KGDH), PDH, ETFDH and aconitase are also known to produce ROS (Zorov et al. 2014). Chronic elevated levels of ROS can be deleterious to cells and tissues by causing oxidative damage to cellular macromolecules and can eventually lead to cell death. Lower concentrations of ROS still have the potential to cause cell damage; however, this damage is typically repaired by cellular defence mechanisms that are discussed in the following sections. In contrast to the largely negative view of cellular ROS as something to be avoided, there is mounting evidence that low concentrations of ROS act as essential signalling molecules required for adaptation and repair (see section 1.1.3.1). Hence, under physiological conditions, cellular mechanisms aim to maintain a tight balance between ROS-induced signalling and pathological levels of ROS.

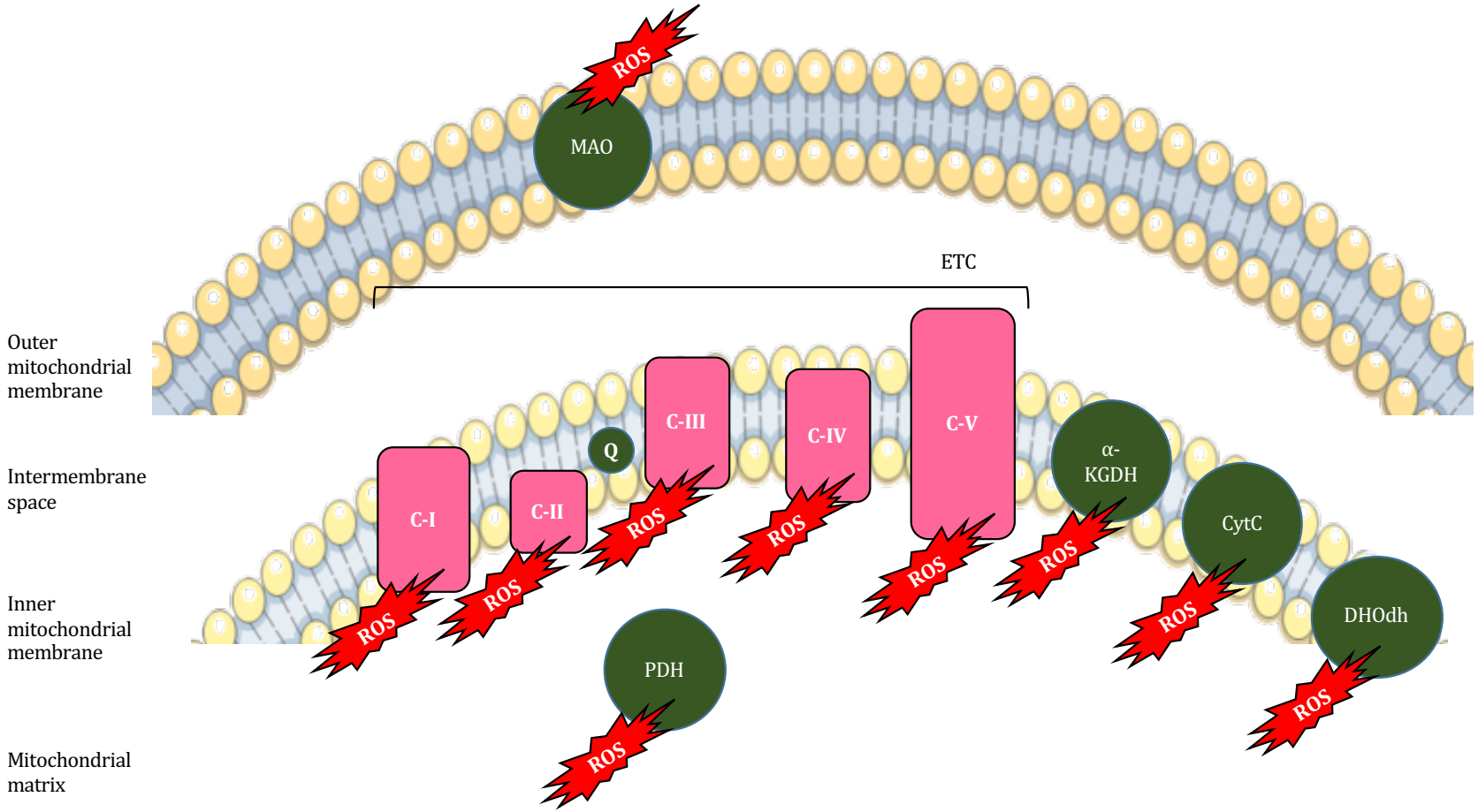


Figure 9: Sites of mitochondrial reactive oxygen species (ROS) generation. Major sites of mitochondrial superoxide ($O_2^{\bullet-}$) generation are complex I (C-I) and complex III (C-III) of the mitochondrial electron transport chain (ETC), while complex II (C-II), complex IV (C-IV) and complex V (C-V) are also involved. Other enzymes like monoamine oxidase (MAO), cytochrome C (Cyt C), α-ketoglutarate dehydrogenase (α-KGDH), pyruvate dehydrogenase (PDH) and dihydroorotate dehydrogenase (DHO-dh) are also responsible for ROS generation.

1.1.3.1 Regulatory role of mitochondrial ROS

For a long time, ROS were generally believed to be detrimental to cellular function. However, recent evidence changes this picture significantly. Mitochondrial ROS, at elevated, but sub-lethal concentrations play an important role as cell signalling molecules that for example regulate cell cycle progression (Holmström and Finkel 2014). It is now established that the redox environment of the cell defines its fate and can induce proliferation, differentiation or even cell death (Ray et al. 2012). Furthermore, different cellular compartments require different redox status for their function. For example, reducing conditions are typically associated with the cytoplasm, while in contrast lysosomes are characterised by an oxidative environment (Alberts et al. 2000).

The mitogen activated protein kinase (MAPK) cascade includes apoptosis signal-regulated kinase 1 (ASK1) that is activated under stressful conditions (including oxidative stress). In resting state, ASK1, through its C-terminal coiled domain forms a homo-oligomer. In the presence of oxidative stress, ASK1 undergoes a conformational change and an additional interface is pre-formed on the existing oligomer that leads to its auto-phosphorylation, which activates ASK1 (Tobiume et al. 2002)

The redox protein, thioredoxin, in its reduced form interacts with ASK1 and inhibits its subsequent activation (Fujino et al. 2007). In the presence of low levels of oxidative stress, ASK1 is activated and induces p38 signalling, which leads to non-apoptotic outcomes such as cell differentiation (Choi et al. 2011) and immune signalling (Matsuzawa et al. 2005).

Phosphoinositide-3-kinase (PI3K) is another signalling pathway that plays a major role in cell proliferation and stimulation by growth factors. Endogenously generated ROS can activate PI3K similar to the MAPK pathways by oxidation, which ultimately results in sustained activation of the signalling pathways (Kwon et al. 2004).

Low doses of H_2O_2 can also promote the release of Ca^{2+} and therefore increase cellular calcium signalling (Giorgio et al. 2017). Furthermore, H_2O_2-mediated lipid peroxidation was demonstrated to activate different isoforms of phospholipases in the mitochondria, cytosol and liposomes. Although the precise mechanism is so far unknown, oxidised lipids are proposed to act as a substrate to phospholipase-C and phospholipase-A that can further increase the release of Ca^{2+} from the endoplasmic reticulum (Domijan et al. 2014). Calcium signalling is also evoked by the generation of $O^{2\cdot-}$ under conditions of mild hypoxia where the mitochondrial ETC is inhibited (Song et al. 2017). These increased cytoplasmic Ca^{2+} levels can induce the translocation and binding of phospholipase to the OMM that leads to the release of Cyt C and other proapoptotic factors from the mitochondria, which initiates cell death (Domijan et al. 2014, Halliwell 2006, van Rossum et al. 2004). The indirect release of Ca^{2+}, mediated by ROS-activated phospholipase isoforms also plays an important signalling role in the brain. The neurotransmitter dopamine also induces the release of intracellular calcium as a secondary event. In this system, the mitochondrial enzyme MAO metabolises dopamine which releases H_2O_2 in the process. This in-turn results in lipid peroxidation, activation of phospholipases and generating IP3-dependent calcium signalling (Vaarmann et al. 2010).

Another important role of ROS includes the regulation of cardiomyocyte (Li et al. 2012) and neuron excitability (Angelova and Müller 2006) by modulating gating properties of various ion channels (Tang et al. 2001). ROS can oxidise cysteine and methionine residues

of ion channels and their associated receptor protein subunits, which ultimately modulates the excitability of muscles and neuronal cells (Kolbe et al. 2010). ROS-induced oxidation of cysteine residues in ion channels can also disrupt calcium-dependant activation of those channels, which therefore can impair muscle vascular tone, responsible for some vascular disorders (Donoso et al. 2011, Tang et al. 2004). In neurons, calcium flux influences neurotransmitter release, plasticity of neurons and neurogenesis (Thanawala and Regehr 2013). Mitochondrial ATP along with TCA cycle intermediates act as building blocks for the synthesis of neurotransmitters including glutamate and γ-amino butyric acid (GABA) (Wallace et al. 2010). Hence, compromised oxidative metabolism coupled with increased oxidative stress is likely to alter neurotransmitter levels and affect brain function.

1.1.3.2 Cellular damage due to oxidative stress

Oxidative stress is the excessive generation of free radicals in biological systems to the extent at which cellular protective mechanisms fail to rescue, leads to the perilous alterations of cellular lipids and proteins. Mitochondria generate ROS as a by-product of OXPHOS. However, mitochondrial dysfunction exacerbates the generation and accumulation of ROS (Zorov et al. 2014). Accumulation of exogenous toxic molecules in mitochondria also lead to the generation of ROS (Zorov et al. 2014). Although ROS can act as cellular signalling molecules, they are harmful to cells after chronic exposure or higher levels. The $HO^{\cdot}$ generated from H_2O_2 can react with almost any macromolecule in its vicinity. The $O^{2\cdot -}$ can attack the double bonds in unsaturated fatty acids and proteins, producing lipid peroxides that ultimately destabilize membranes (Schumacker 2015).

Free radicals, unlike normal cellular substrates do not depend on enzymatic activity of cells, but are aggressive, highly reactive molecules involved in spontaneous reactions (Figure 10) (Turrens 2003). Cellular macromolecules such as lipids, proteins and DNA are highly susceptible to ROS-induced damage. Damage to proteins would reduce their affinity towards enzymes, coenzymes and substrates and will ultimately affect protein function (Bandyopadhyay et al. 1999, Klaunig et al. 2010). Accumulation of mitochondrial ROS is implicated in many pathological conditions including neurodegenerative disorders (Feitosa 2018, Hatziagapiou et al. 2018). ROS-induced oxidative damage to mitochondria functions as a vicious cycle, where the resultant damage to ETC complexes further exacerbates the generation of ROS causing additional damage to cell (Zorov et al. 2014). For example, free radicals generated by C-I and C-III of the mitochondrial ETC have access to the mitochondrial membrane. These radicals can oxidise the phospholipids on the mitochondrial membrane. The resulting formation of lipid peroxides derails the composition, fluidity and barrier function of the mitochondrial membrane. The addition of such polar, peroxyl groups also modifies the arrangement of fatty acids in the membrane, which increases its permeability and can ultimately leading to cell death (Kagan et al. 2005).

Cardiolipin, an anionic phospholipid, is found primarily on the IMM bound to Cyt C. Cardiolipin has a unique structure among phospholipids by containing four acyl chains in contrast to most lipids that are composed of only two acyl chains. It is required for the stability and fluidity of the mitochondrial membrane, but also for the formation of mitochondrial cristae (Mannella 2006, Schlame et al. 2000). In the presence of oxidative stress, cardiolipin is oxidised by cardiolipin peroxidase and results in the mobilisation of Cyt C from the IMM. Decrease in intact cardiolipin content leads to increased membrane permeabilisation, which provides access for proapoptotic factors in to the mitochondria

along with the release of Cyt C as a key feature for the induction of apoptosis (Kagan et al. 2005). Apart from its role in mitochondrial membrane integrity, cardiolipin is also shown to bind to the ETC complexes, C-I, C-III, C-IV, whose activity is decreased in the absence of cardiolipin. Cardiolipin facilitates the association of mitochondrial C-III and C-IV into super-complexes that is believed to improve OXPHOS efficiency (Zhang et al. 2002). The presence of super-complexes eliminates the need for substrates to diffuse substrates between individual components of the mitochondrial ETC. Hence, in the presence of oxidative stress, the activity of ETC complexes is directly impaired by cardiolipin oxidation apart from increasing membrane permeabilisation and inducing the release of proapoptotic factors (Bhat et al. 2015).

mtDNA is significantly more susceptible to oxidative damage since it is not protected by histones, lacks introns and has DNA repair mechanism that are less effective compared to nuclear DNA (nDNA) (Lenaz 1998, Chan 2006). Therefore, prolonged exposure of mitochondria to non-physiological concentration of ROS significantly increases the likelihood of mutations in the mtDNA (Wenzel et al. 2008). These mtDNA mutations, that constitute point mutation and deletions of different length will not only decrease the mitochondrial oxidative phosphorylation capacity but also increase the generation of ROS even further (Zorov et al. 2014).

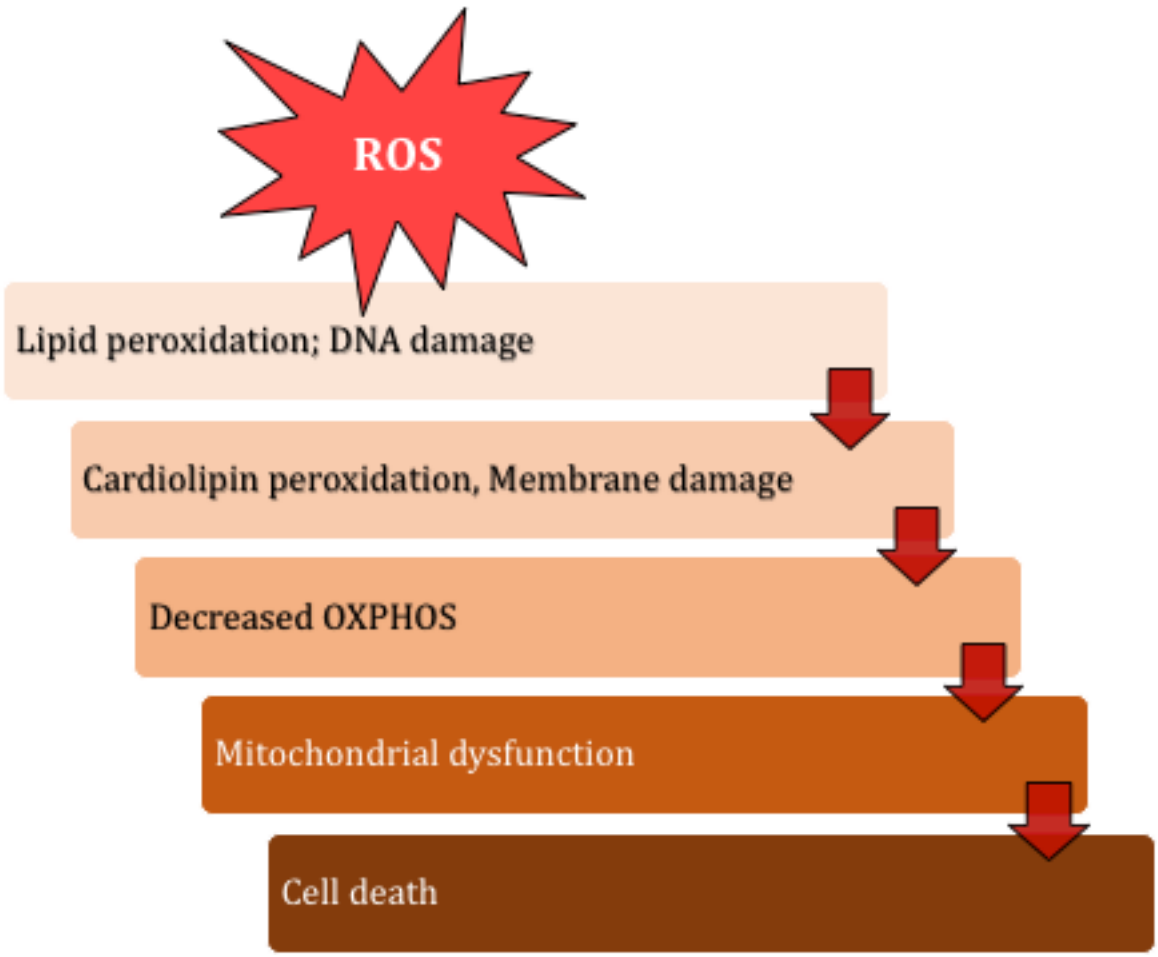


Figure 10: Damage due to reactive oxygen species. Reactive oxygen species (ROS) are highly aggressive molecules and can involve in spontaneous reactions. ROS can damage DNA and cause alterations to proteins, lipids causing cardiolipin peroxidation and membrane damage. These lead to decreased mitochondrial oxidative phosphorylation (OXPHOS) which in turn causes mitochondrial dysfunction. All these processes can ultimately lead to cell death.

1.1.4 Cellular defence mechanisms against ROS-induced damage

ROS, generated as a by-product of cellular aerobic metabolism, can be extremely detrimental to cells at higher concentrations. To counteract oxidative stress, cells have developed or use defence mechanisms involving a plethora of enzymatic antioxidants

such as SOD, catalase, glutathione-peroxidase, peroxiredoxins (Lebovitz et al. 1996, Skulachev 1996) and small-molecule antioxidants including vitamins such as β-carotene (vitamin A), ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (Do et al. 2015, Kasperczyk et al. 2014).

SOD detoxifies $O^{2\bullet-}$ and is present as two distinct cellular isoenzymes in different cell compartments. Cytosolic SOD is coupled with zinc and copper atoms (Zn-SOD, Cu-SOD) as cofactors (Okado-Matsumoto and Fridovich 2001) in contrast to mitochondrial SOD, which utilises manganese (Mn-SOD) (Govatati et al. 2016). SOD reacts with $O^{2\bullet-}$ and converts it into H_2O_2. Although not a free radical itself, H_2O_2 is sufficiently reactive to be damaging and in the presence of ferrous ions and generates $HO^{\bullet}$. In addition, H_2O_2 is able to easily cross membranes which makes this molecule a potent toxin at higher concentrations (Murphy 2009). Cells therefore detoxify H_2O_2 by a catalase-dependent reaction that converts it into water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is located mainly in the peroxisomes. In addition to catalase, other enzymes classified as peroxidases, like glutathione peroxidase (GPx1) are common cytoplasmic enzymes that detoxify H_2O_2. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is one of the most abundant cellular antioxidants. It is the major mitochondrial ROS-detoxifying agent due to its high millimolar intra-mitochondrial concentrations (Montero et al. 2009). The GSH tripeptide which is linked with a γ-bond is claimed to be resistant against degradation reactions (Sheehan et al. 2001). In the presence of pro-oxidants, the sulphydryl group of GSH, which is the redox active moiety, is oxidised to glutathione disulphide (GSSG), a homodimer of GSH. As a result, H_2O_2 is converted to water in the presence of GPx1 (Figure 11) (Nelson et al. 2008). However, the sulphydryl group can

also react with the cysteine residue on proteins to form PSSG (glutathionylated protein) that often results in inhibition of their activity or changes in protein stability (Ghezzi 2013).

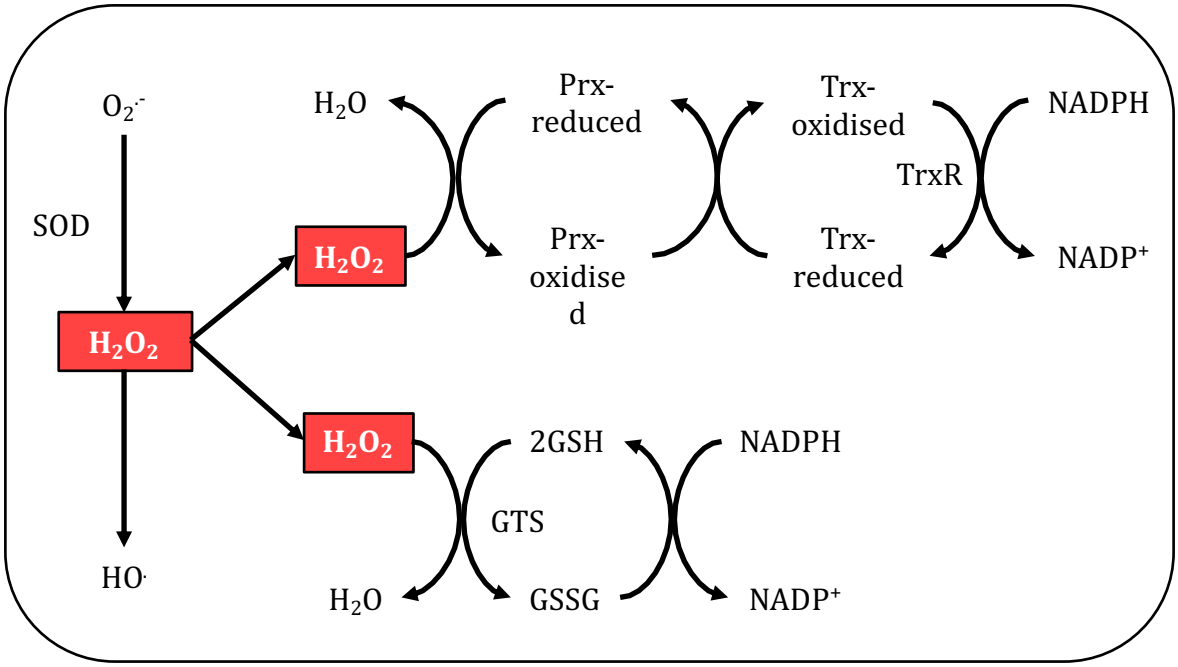


Figure 11: Cellular defence mechanisms. Due to the high reactivity of $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2) is generated by superoxide dismutase (SOD). The resultant H_2O_2 is further detoxified to water (H_2O) by glutathione peroxidase (GPx1) utilising reducing equivalents from the oxidation of glutathione (GSH) to glutathione-disulphide (GSSG) in the presence of GSH-reductase (GR). H_2O_2 can also be detoxified by peroxiredoxin (Prx) pathway that is further reduced by utilising electron equivalents from thioredoxin (Trx) in the presence of Trx-reductase (TrxR). Both GSH and Trx can be further regenerated by utilising electron equivalents from reduced form of nicotinamide adenine dinucleotide phosphate (NADPH).

Intracellular GSH is regenerated from GSSG by the enzyme GSH reductase (GR) by using NADPH from PPP (Nelson et al. 2008). In fact, GSH is also involved in the reactivation of vitamin C, ascorbic acid from dehyro-ascorbic acid (Meister 1992).

$$\text{Dehydroascorbic acid} + 2\text{GSH} \rightarrow \text{Ascorbic acid} + \text{GSSG}$$

Another class of enzymes, peroxiredoxins (Prx) are involved in detoxifying H_2O_2 in the presence of thioredoxin (Trx) and the enzyme thioredoxin reductase (TrxR) (Figure 11) (Perkins et al. 2015).

Cyt C, located on the inner mitochondrial membrane acts as an essential electron carrier in the mitochondrial ETC. This form of Cyt C can detoxify $O_2^{\cdot -}$ and H_2O_2 converting it into molecular oxygen and water. The reduced Cyt C is then re-oxidised by C-IV of the ETC providing electrons to the final step of ETC (Pereverzev et al. 2003, Skulachev 1998).

1.1.5 Quality control of mitochondria

In general, mitochondrial energy production is regulated tightly in line with other metabolic activities such as maintaining redox homeostasis and supplying intermediates for protein and DNA synthesis. For this purpose, mitochondria undergo not only metabolic but also morphological changes depending on the cellular environment. Mitochondrial network fragmentation (mitochondrial fission) is promoted by the oversupply of substrate, in contrast to substrate shortage, which promotes mitochondrial elongation (mitochondrial fusion) (Gomes et al. 2011, Molina et al. 2009).

Dynamin-related-protein-1 (Drp1), a large dynamin-related GTPase regulates mitochondrial fission (Hoppins et al. 2007). Fission occurs when Drp1, located in the cytosol translocates to the OMM and binds to the integral OMM proteins Fis-1, Mff and MIEF1, followed by post-translational modifications. However, the exact mechanism of this interaction is not fully understood yet (Gottlieb and Bernstein 2016). Drp-1, has recently been reported to also play a role in cristae remodelling and result in dramatic fragmentation of mitochondria (Große et al. 2016).

Mitochondrial fusion is essential to allow the exchange of intermembrane space and matrix contents including the mtDNA, between mitochondria (Chen et al. 2007). Mitochondrial fusion is mediated by mitofusins (MFN1, MFN2) and optic atrophy-1 (OPA1) proteins located in the OMM and IMM respectively. Mitofusins promote mitochondrial fusion by the formation of dimers by activating GTPase and in the presence of OPA1. In the absence of MFN2, another protein named mitochondrial elongation factor-1 (MIEF1) promotes the fusion of mitochondria (Gottlieb and Bernstein 2016, Zhao et al. 2013).

Mitochondrial fusion and fission, as key morphological responses are collectively controlled by energy status, the activity of mitochondrial ETC, environmental factors such as toxins and cellular kinases (Mishra and Chan 2016). Impaired processes of mitochondrial fusion or fission lead to mitochondrial dysfunction and are responsible for a variety of degenerative disorders (Reddy 2014, Wang et al. 2009). Exposure of cultured cells to stress in the form of toxins or ultraviolet (UV) rays promotes mitochondrial fission, which is balanced by mitochondrial fusion in order to improve cellular viability. Inhibition of mitochondrial fusion can lead to loss of mtDNA, OXPHOS deficiencies and a significantly increased ROS levels (Chen and Chan 2009). Defects in Drp-1 are associated with cardiomyopathy and in vivo lethality (Ashrafian et al. 2010) whereas mutations in mitofusins and OPA1 cause neurodegenerative diseases such as dominant optic atrophy respectively (Alexander et al. 2000, Züchner et al. 2004).

Under conditions of high-energy demand, transient hypoxia or a lack of respiratory units (ETC complexes) result in energy deficit. In these scenarios, mitochondrial biogenesis (mitogenesis) and cellular glycolysis are typically upregulated. A coordinated synthesis and assembly of both nuclear and mitochondrial encoded subunits of the ETC complexes

are required for mitogenesis. Nuclear-encoded proteins are translated by cytoplasmic ribosomes and are subsequently imported into the mitochondria. This process is governed by the mitochondrial membrane potential hence facilitating the assembly of both mitochondrial and nuclear encoded subunits into functional complexes that constitute the ETC (Jornayvaz and Shulman 2010). Specific assembly factors like nuclear respiratory factor 1 and 2 activate mitochondrial transcription factor that allows the replication of mtDNA. Excess or unincorporated subunits are degraded by proteases in the mitochondrial matrix (Palikaras et al. 2015). The major regulator of mitogenesis belong to peroxisome proliferator-activated receptor gamma (PPARγ) family, mainly PPARγ coactivator-1-alpha (PGC-1α), which is upregulated in response to increased energy demands (Lehman et al. 2000). PGC-1α is activated by exogenous cues such as stress, ROS, calorie restriction that in-turn increases the transcription of genes involved in metabolic pathways including mitogenesis (Fernandez-Marcos and Auwerx 2011). Cellular kinases such as p38 MAPK, the homologue of silent information regulator protein (SIRT1) as well as the energy sensor AMP-activated protein kinase (AMPK) induce PGC-1α expression and enhance its activation when ATP levels are low (Reznick et al. 2007).

In contrast to mitogenesis, an autophagic process called mitophagy, removes defective mitochondria. Following the release of signals like ROS, DNA damage etc., the mitochondrial outer membrane becomes permeable to Cyt C, endonucleases apart from other proteins in the intermembrane space that activate caspases to carry out apoptosis. Mitophagy is a degradation process that effectively eliminates damaged mitochondria from healthy ones (Youle and Narendra 2011). Mitophagy not only removes defective mitochondria that harbour risk of excess ROS production, but also provides an additional source of energy via the metabolic use of energy-rich amino acids (Kim et al. 2007). Mitophagy is regulated by cellular kinases such as PTEN-induced putative kinase-1

(PINK1) located on the OMM and a cytoplasmic ligase, parkin. Other proteins such as Nix and BH-3-proapoptotic proteins (Bnip3) act as receptors by binding to LC3 located on the autophagosome that aids targeting mitochondria to the autophagosome (Youle and Narendra 2011).

Permeabilisation of the mitochondrial outer membrane occurs by the opening of mitochondrial permeability transition pore (mPTP) (Bernardi 1999, Mnatsakanyan et al. 2017). The mPTP permits the passage of molecules smaller than 15kDa and is sensitive to intracellular calcium homeostasis (Elrod and Molkentin 2013). This pore allows entry of metabolites into the mitochondrial matrix. However, prolonged opening of the mPTP may lead to osmotic dysregulation in the inner mitochondrial membrane, leading to uncoupling of the electron transport system from ATP synthase which leads to reduced ATP generation and ultimately resulting in cell death (Mnatsakanyan et al. 2017). In cultured cells, upon induction of apoptosis (by toxins like actinomycin or UV radiation), pores in mitochondrial outer membrane open to release Cyt C into the cytosol, followed by decreased ETC activity, suppression of respiration rate, decreased membrane potential, cleavage of OPA1 and disassembly of cristae junctions that ultimately lead to cell death. However, in the presence of caspase inhibitors, apoptosis is blocked (Burke 2017). Collectively, mitochondrial fission, fusion, mitogenesis and mitophagy regulate the morphology of mitochondria and is referred to as mitochondrial dynamics (Chen and Chan 2009).

1.2 Mitochondrial dysfunction and disease

Mitochondria are multi-functional organelles, serving many roles that involve energy generation, Ca^{2+} homeostasis, intermediate biosynthesis, regulation of cell death that in summary determine cellular survival. Therefore, not surprisingly, dysfunctional mitochondria are implicated in a large range of diseases that can affect nearly every tissue in either children or adults (Figure 12). Although it would be tempting to connect specific pathological conditions to specific mitochondrial pathways, the current literature suggests a more complex scenario. At present, there is good evidence that mitochondrial dysfunction-induced disorders involve multiple interrelated pathways and mechanisms such as oxidative stress, calcium dysregulation, age-related and inherited DNA mutations that cause mitochondrial dysfunction (Boczonadi and Horvath 2014, Burté et al. 2015).

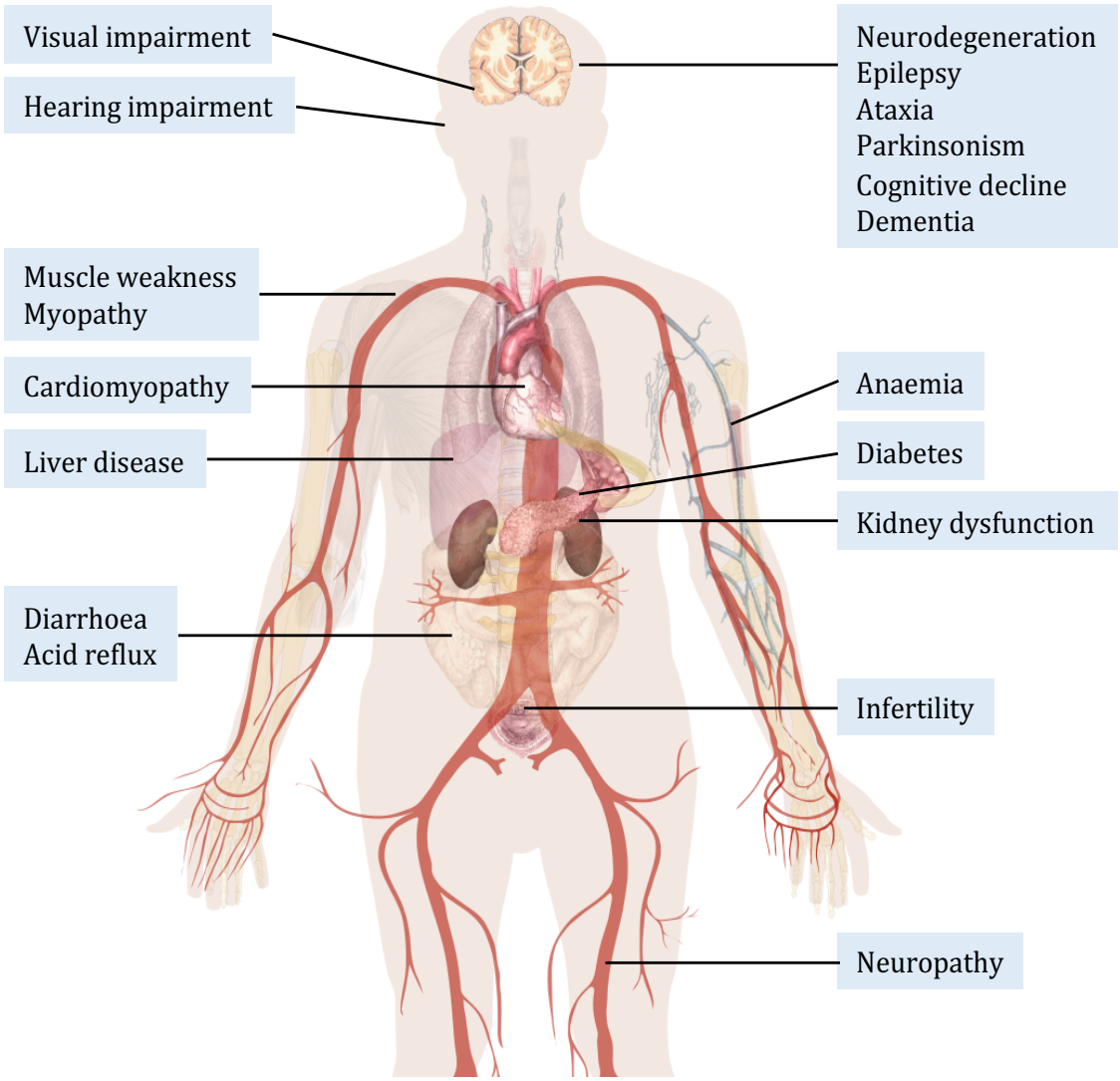


Figure 12: Clinical complexity of mitochondrial diseases. Mitochondrial dysfunction can affect any organ in the body either solely or as a multi-system disorder.

1.2.1 Diseases due to mitochondrial dysfunction

Genetic disorders with a mitochondrial phenotype could be caused either due to mutations in mitochondrial genes (mtDNA or nDNA) or by the impairment of mitochondrial function is impaired by external factors (i.e. drugs, oxygen deprivation, ROS etc.). For normal function, mitochondria require more than 1500 proteins, of which only 13 proteins are encoded by the mtDNA while the vast majority of proteins are encoded in the nucleus (Neupert and Herrmann 2007). These nuclear encoded proteins are translated in the cytoplasm before being imported into the mitochondria. Since mitochondria are solely maternally inherited, mutations in mitochondrial genome do not follow classic Mendelian rules of inheritance (Al Rawi et al. 2011, Politi et al. 2014). Each cell contains several hundreds to thousands copies of mtDNA that may differ in sequence, hence referred to as heteroplasmy (Robin and Wong 1988, Satoh and Kuroiwa 1991, Sender et al. 2016). It is believed that during mitosis, the distribution of mitochondria into daughter cells occurs randomly, which can accumulate specific mtDNA mutations to different extent in specific cells that can include oocytes (Sproule and Kaufmann 2008). As a result, the accumulation of a mutation in any specific cell occurs incidentally and the same mutation might not manifest itself in the same way in two carriers (Sproule and Kaufmann 2008). Therefore, a diverse clinical phenotype is observed in patients with mitochondrial mutation.

Mutations in mtDNA can lead to an extensive array of symptoms that can affect nearly all tissues in the body but mainly affects tissues that are characterised by large energy demands. Mutations in mtDNA resulting in primary mitochondrial diseases either encode for OXPHOS proteins directly or impact their function by altering the production of

machinery required to run OXPHOS. The pathological symptoms of mitochondrial dysfunction typically include blindness and other vision disorders, deafness, diabetes, muscle atrophy, exercise intolerance and impaired cognitive function. These symptoms can sometimes show a significant degree of overlap between different diseases such as Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like Episodes (MELAS) or optical neuropathies like Leber's hereditary optic neuropathy (LHON), neuropathy, ataxia and retinitis pigmentosa (NARP) and macular degeneration, which complicates diagnosis based on patient characteristics alone (Table 1) (Sproule and Kaufmann 2008). Therefore, diagnosis is predominantly based on detection of characteristic disease-specific mutations and DNA modifications. For example, specific mtDNA deletions are observed in Kearns-Sayre syndrome and Pearson syndrome (Wong 2001), while mutations in genes encoding ribosomal RNA can cause aminoglycoside-induced non-syndromic deafness (Zhu et al. 2006).

Mutations in nuclear-encoded genes that encode for mitochondrial proteins can also lead to disorders with a mitochondrial phenotype. For example, Friedreich's ataxia (FRDA), a neurodegenerative disease is caused by the loss of frataxin protein that localizes in the mitochondria and is involved in the iron-sulphur cluster biosynthesis within mitochondria (Vanlander and Van Coster 2018). Point mutations in nDNA (NDUFS1, SURF1) can cause Leigh syndrome (LS) (Martín et al. 2005).

Table 1: Some representative examples of mitochondrial diseases

Disease	Mutations in		Disease features	References
	mtDNA	nDNA		
LHON	✓		Acute loss of visual acuity	(Gueven 2014, Catarino et al. 2017)
MELAS	✓		Encephalomyopathy, lactic acidosis, stroke-like episodes	(Lin et al. 2017)
MERRF	✓		Myoclonic epilepsy, myopathy, ataxia, dementia	(Hirano et al. 2006, Chiu et al. 2017)
LS	✓	✓	mtDNA effect: Cardiomyopathy, grey and white matter abnormalities nDNA effect: Cardiomyopathy, encephalomyopathy, hepatic dysfunction	(Thorburn et al. 2017)
ANS	✓		Sensory ataxia neuropathy, ophthalmoplegia, epilepsy, cerebral ataxia	(Ahola et al. 2014)
FRDA		✓	Ataxia, cardiomyopathy, optic atrophy, diabetes mellitus	(Abeti et al. 2016)
Kearns-Sayre syndrome	✓		Pigmentary retinopathy, heart block	(Kabunga et al. 2015)

LHON, Leber's hereditary optic neuropathy; MELAS, Mitochondrial encephalomyopathy with lactic acidosis and stroke like episodes; MERRF, Myoclonic epilepsy and ragged red fibres; AD, Alzheimer's disease; LS, Leigh syndrome; ANS, Ataxia neuropathy spectrum; FRDA, Friedrich's ataxia.

1.2.2 Diseases with mitochondrial dysfunction

Many inherited or acquired diseases are also associated with deletions in mtDNA that are secondary to the actual disease pathology (i.e. occurs much later in the disease) hence leading to mitochondrial dysfunction, which can affect the ability to generate ATP or even the mitochondrial dynamics. For example, multifactorial diseases such as diabetes (Archer 2013), cardiac (Ong et al. 2013) and kidney diseases (Zhan et al. 2013) are believed to be associated with mitochondrial dysfunction and also affect the fission and fusion of mitochondria.

A range of myopathies including muscular atrophy, spinal muscle atrophy (SMA), inflammatory myopathies, drug-induced peripheral neuropathies (Katsetos et al. 2013) involve mitochondrial dysfunction (Table 2). mtDNA depletion was shown in molecular studies of muscle samples from neuromuscular diseases (Komulainen et al. 2015). In diseases like Down syndrome, chromosomal rearrangements can disrupt nuclear genes that encode for mitochondrial proteins, which can cause secondary mitochondrial dysfunction (Valenti et al. 2011, Pagano and Castello 2012). Mitochondrial dysfunction has also been noted in autoimmune disorders like lupus (Morris et al. 2014, Morris et al. 2015), pemphigus vulgaris (Feichtinger et al. 2014). In fact, mitochondrial dysfunction is also implicated in children with neurodevelopmental disorders and autism spectrum disorders (Rossignol and Frye 2012; Rose et al. 2014).

On the other hand, mutations in the genes encoding for mitochondrial proteins are also linked to some types of cancer. For example, mutations in C-II of ETC have been linked to cancers like paraganglioma and pheochromocytoma (Baysal et al. 2000, Astuti et al. 2001).

Table 2: Some representative examples of diseases with mitochondrial dysfunction

Disease	Tissue affected	Disease features	References
SMA	Spinal cord	Hypotonia, muscle weakness, loss of motor neurons	(Ripolone et al. 2015)
Down syndrome	Multisystem disorder	Hypotonia, epicanthal folds cognitive impairment, hearing loss	(Valenti et al. 2014)
ASD	CNS, immune system, endocrine	Sensory modulation symptoms	(Hollis, et al. 2017)
AD	CNS	Slow, progressive loss of cognition, memory and intellectual function	(Chhetri et al. 2018)
PD	Multisystem disorder	Tremor, loss of motor, cognitive and limbic function	(Ryan et al. 2015)
MS	CNS	Demyelination, progressive axonal degeneration, loss of visual acuity	(Patergnani et al. 2017)

SMA, spinal muscle atrophy; ASD, autism spectrum disorder; CNS, central nervous system; AD, Alzheimer's disease; PD, Parkinson's disease; Multiple sclerosis

Mitochondrial dysfunction can also result as a consequence of exposure to environmental stressors and xenobiotics not related to the mitochondria per se. Oxidative damage to brain mitochondria of rats decreased the activities of C-IV and C-V of ETC, apart from a decrease in binding affinity, coupled with reduced activity in C-I (Long et al. 2009). These effects lead to mitochondrial dysfunction that can ultimately accelerate the ageing process (Long et al. 2009). Mitochondrial membrane disruption as a result of dysregulated calcium homeostasis is believed to result in Duchenne Muscular Dystrophy (DMD) (Allen and Whitehead 2011). Mitochondrial toxicity has been shown for some drugs such as statins, that are used to treat hyper-cholersterolemia are known to induce myopathy by depleting tissue CoQ10 stores (Joy and Hegele 2009). For example, high levels of ROS induce alterations in the differentiation and number of CD4 cells that contribute to the development of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS) (Solleiro-Villavicencio

and Rivas-Arancibia 2018). Many other drugs such as antibiotics, inhalation narcotics and non-steroid anti-inflammatories are also long known to induce mitochondrial dysfunction (Chan et al. 2005, Labbe et al. 2008).

Mitochondrial dysfunction can also be caused due to metabolic dysregulation. Deficiency in micronutrients accelerates mitochondrial decay since the mitochondrial function is supported by enzymes, cofactors and metabolites synthesised from vitamins and minerals (Ames 2010). Elevated mitochondrial iron levels also decrease mitochondrial activity by increasing oxidative stress (Liddell and White 2017).

To stay within the scope of the thesis, only one mitochondrial disease, LHON, is discussed in detail in the next section.

1.2.3 Leber's hereditary optic neuropathy

LHON, although a rare disease, it is one of the most common mitochondrial disorders with an incidence of approximately 1:45,000 in Europe (Mascialino et al. 2012), 1:31,000 people in North-East England (Man et al. 2003) and 1:50,000 in Finland (Puomila et al. 2007). In about 95% of the cases, LHON is caused by three characteristic mutations (m.11778G>A in the ND4 gene or m.3460G>A in the ND1 gene or m.14484T>C in the ND6 gene) (Howell 1998, Pilz et al. 2017). In the remaining 5 % of the cases, mutations in ND5 gene (m.13730AG>A), ND6 gene (m.14459G>A, m.14482C>G, m.14495A>G, m.14498C>T, m.14568C>T and m.14596T>A) have been described (Man et al. 2002). Recently, additional mutation in ND4 gene (m.10663T>C) was reported in a case study (Jørstad et al. 2018). Apart from primary mutations in mtDNA, the individual genetic haplotype in mitochondria also play a huge role in the onset and severity of the disease (Hudson et al.

2007). In individuals with haplogroup J, mutations in m.11778G>A and m.14484T>C were found to be associated with a significantly higher risk of vision loss whereas haplogroup H appeared to protect individuals with the m.11778G>A mutation (Hudson et al. 2007).

Intriguingly, only about 50 % of males and 10 % of females that harbor mutations in these genes, actually develop optic neuropathy (Man et al. 2002). This incomplete penetrance is not understood at present. While environmental risk factors such as smoking and alcohol are thought to be implicated as they can dose-dependently elevate the energy deficit either directly by compromising the activity of C-I or indirectly by reducing arterial oxygen content (Giordano et al. 2015, Kirkman et al. 2009), there is some experimental evidence in vitro that oestrogen could have neuroprotective effects (Giordano et al. 2010). This observation could at least partially explain the significantly lower risk to develop retinopathy observed in female mutation carriers. Overall, the incomplete penetrance indicates that the disease onset likely involves a plethora of interacting factors that apart from mitochondrial dysfunction, energy deficiency, excess ROS include secondary genetic, hormonal, anatomical, physiological variations and/or environmental factors play a significant role in disease development (Man et al. 2002).

LHON mutation carriers are typically asymptomatic until they experience rapid unilateral clouding or blurring of vision in one eye followed by acute vision loss in one eye. Simultaneously, loss of visual acuity is seen in the other eye within weeks or months (Yu-Wai-Man et al. 2009, Fraser et al. 2010). However, in some rare cases, second eye involvement was reported to be as long be delayed up to 18 years (Milea et al. 2010, Ohden et al. 2016). Decreased visual acuity, 4-6 weeks after onset can reduce to 6/60 or

worse and is typically accompanied by marked impairment in colour vision (Yu-Wai-Man et al. 2011).

The disease progression of LHON can be divided into three stages. The first, pre-symptomatic phase of LHON is characterised by peripapillary angiopathy without affecting visual acuity. The second, acute phase of the disease is initiated by LHON mutations in combination with secondary genetic and environmental factors. This phase is typically characterised by loss of visual acuity which is also associated with the swelling of retinal nerve fibre layer (RNFL), vascular tortuosity of retinal vessels and circumpapillary telangiectatic microangiopathy, energy deficiency and oxidative stress. (González et al. 2017). In some patients, increased intraocular pressure was also observed, similar to glaucoma (Abu-Amero et al. 2006, Thouin et al. 2013). As a result of these cellular pathologies, the retinal ganglion cells (RGC) become dysfunctional and are unable to transmit information to the brain despite being alive. The loss of RGCs occurs in the final, atrophic phase where the optic nerve is functionally impaired leading to atrophy of the nerve (Barboni et al. 2010, Barcella et al. 2010). During the chronic phase of the disease, increasing deterioration of visual acuity in most cases eventually leads to blindness (Brown et al. 2001). This is supported by histological post-mortem analysis of optic nerve of LHON patients that shows degeneration of RGC axons largely limited to the central, inferonasal sector (Saadati et al. 1998). This three-phase model indicates a window of opportunity that includes viable but dysfunctional cells during which spontaneous recovery could be possible. In some rare patients, spontaneous vision recovery has been described in the first year after disease onset. This phenomenon was even seen in some patients after several years (Stone et al. 1992), depending on the underlying mutation. Between one to four years after disease onset, the lowest

spontaneous recovery rates (15.4%) were observed in patients with the m.3460G>A mutation. In contrast, patients with m.11778G>A and m.14484T>C mutation that exhibited intermediate (22%) and highest (>50%) chances of recovery respectively (Spruijt et al. 2006). Although, the underlying mechanisms of spontaneous recovery of vision are not understood, it nevertheless indicates the possibility to employ potential therapeutic approaches in this time window to restore visual acuity in patients after disease onset.

Although LHON is uniquely characterised by loss of vision, patients sometimes also present with cardiac arrhythmias, myopathy, encephalopathy, epilepsy, dystonia, psychiatric disturbances (Chalmers and Harding 1996, Finsterer et al. 2017) and in some very rare cases, a condition characterised as LHON-MS-overlap syndrome (Harding et al. 1992). Vitamin B12 deficiency was also observed in a case study of three patients with LHON; however, the role of B12 in development of LHON is unknown (Pott and Wong 2006).

Biochemical defects in LHON

All major LHON mutations result in amino acid changes in the subunits of C-I of mitochondrial ETC. Mitochondrial C-I dysfunction is connected to decreased membrane potential, reduced ATP synthesis as a result of compromised OXPHOS (Barrientos and Moraes 1999). Phosphorous magnetic resonance spectroscopy studies carried out in whole body of LHON patients have reported maximum reduction in mitochondrial ATP synthesis in LHON with m.11778G>A mutation, followed by m.14484T>C and m.3460G>A (Lodi et al. 2002). Transmitochondrial cybrids carrying one of the three

LHON mutations also showed C-I-dysfunction-induced oxidative stress (Carelli et al. 2004, Floreani et al. 2005, Wong et al. 2002).

Compromised OXPHOS is also associated with elevated levels of ROS (Carelli et al. 2004). This was supported by results from *in vitro* LHON cybrids that harbour one of the primary LHON mutations. Consistent with these data, blood leucocytes of LHON patients showed elevated levels of biomarkers of oxidative stress (Chen et al. 2004, Klivenyi et al. 2001). LHON cybrids also have impaired excitatory amino acid transporter-1 activity that is crucial for glutamate uptake into the muller cells of the inner retina, hence resulting in increased excitotoxic injury to RGCs (Baracca et al. 2005, Beretta et al. 2004). Increased ROS and glutamate excitotoxicity can lead to RGC dysfunction hence impairing the transmission of optic signals to the brain. Exposure to this toxic environment over a prolonged period of time during the acute phase of the disease is believed to lead to the death of RGCs by a specific form of FAS-dependent apoptosis that is independent of caspase (Ghelli et al. 2003). This apoptotic loss of RGC is believed to be responsible for the permanent loss of vision in the final, atrophic stage of LHON.

1.3 Therapeutics approaches for mitochondrial diseases

Despite an increased understanding of the underlying defects of mitochondrial diseases and significant improvements in diagnostic possibilities, unfortunately, no effective treatments have been approved for mitochondrial diseases so far. Treatments of mitochondrial disorders are either disease-specific to correct the underlying defect or aim to alleviate disease symptoms, supplement and/or support mitochondrial function in general. Clinical and molecular features of individual patients determine the use of symptomatic treatments, but this approach can pose significant difficulties to clinicians. For example, a typical approach to treat seizures in paediatric patients includes valproic acid. However, in patients with mitochondrial DNA polymerase gamma (POLG) mutations valproic acid should be avoided as it is associated with significant adverse effects such as severe liver disease in these patients (Cohen et al. 2014). There is some evidence that valproic acid impairs the proton flow through the mitochondrial ETC complexes, which could be the reason that it can exacerbate the symptoms of mitochondrial dysfunction (Lin and Thajeb 2007, Nanau and Neuman 2013).

In this chapter, I focused on recently emerging therapeutic options for treating mitochondrial diseases. One of the general treatment approaches aims to reduce the elevated levels of oxidative stress associated with mitochondrial dysfunction. This can be done directly by using antioxidant molecules or indirectly by strategies to increase the regeneration of endogenous antioxidants such as glutathione (Table 3). Another approach to alleviate mitochondrial dysfunction is by increasing the number of mitochondria by compounds such as bezafibrate and RTA-408 or by upregulating energy generation by molecules such as carnitine and dichloroacetate that supplement substrate supply for energy generation pathways (Table 3). To enhance mitochondrial

accumulation of the drug molecules, compounds such as Mitoquinone, SkQ1 and MTP-131 (Figure 13) have been developed, which have integrated chemical moieties such as the charged triphenyl phosphonium ion or peptides that are known to target specifically to the mitochondria (Table 3). Short-chain quinones, like idebenone (Figure 13) are proposed to target the mitochondrial ETC by bypassing respiratory C-I and ultimately improving energy generation but also act as antioxidants (Table 3).

Dietary modifications and exercise are also suggested as supportive treatment to mitochondrial disorders. Ketogenic diet (KD) is characterised by high fat content combined with low protein and carbohydrate content. There is some limited evidence that KD can beneficially affect respiratory chain defects in patients with MELAS, epilepsy and other diseases associated with dysfunctional OXPHOS (Geffroy et al. 2018). KD is a well-tolerated long-term treatment option not only in pre-clinical disease models (Douris et al. 2015) but also in patients (Bergqvist et al. 2005).

Several small-molecule treatment options for mitochondrial diseases are in development, with some of them in clinical trials (Table 4) and some on the market (Gueven et al. 2017). The following sections focus on the most progressed compounds, to stay within the scope of the thesis. Although the bulk of the data discussed in the following sections is derived from pre-clinical experiments, some drugs have also been assessed in clinical trials. Unfortunately, the results from only few studies are published, while the rest are either ongoing or terminated (Table 4). Wherever available, results from clinical trials are also discussed.

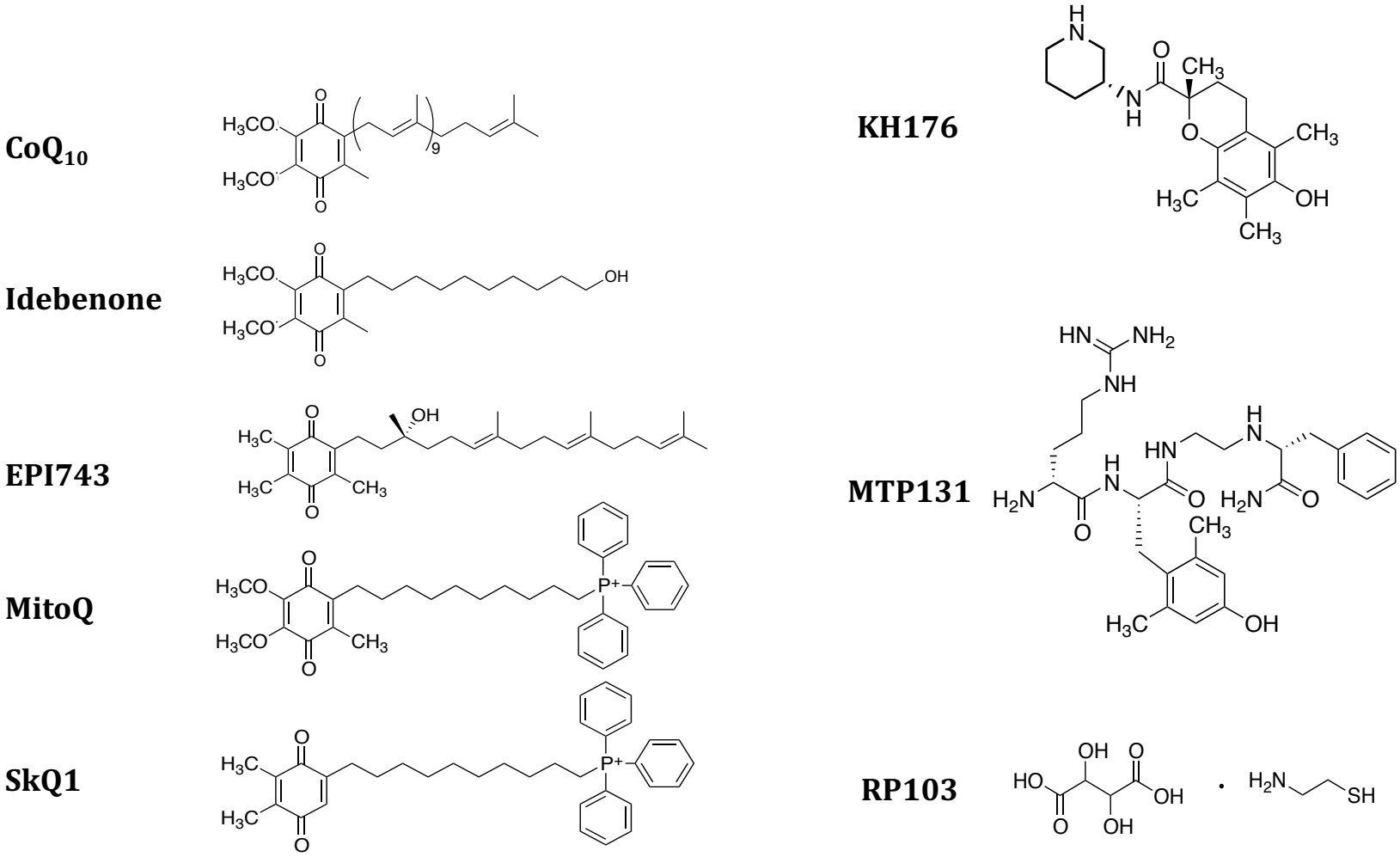


Figure 13: Structures of few drug candidates that are under investigation to treat mitochondrial diseases.

Table 3: Therapeutics for mitochondrial disorders classified based on their mechanism of action

Proposed mode of action	Agents	References
Modification of redox environment	CoQ_{10} Idebenone KH176 α-lipoic acid Dimethylglycine EPI743 RP103 MitoQ SkQ1 MTP-131 Nicotinamide riboside Epicatechin	(Rodriguez et al. 2007) (Erb et al. 2012) (Koene et al. 2017) (Rodriguez et al. 2007) (Bai et al. 2016) (Enns et al. 2012) (Enns and Cohen 2017) (Smith and Murphy 2010) (Anisimov et al. 2008) (Chen et al. 2017) (Khan et al. 2014) (Bettaieb et al. 2014)
Regeneration of glutathione	EPI743 RP103	(Enns et al. 2012) (Enns and Cohen 2017)
Increasing substrate supply	Carnitine Niacin Thiamine Dichloroacetate SRT2104	(Marcovina et al. 2013) (Perumal et al. 2005) (Venhoff et al. 2002) (Stacpoole et al. 2008) (Mercken et al. 2014)
Mitochondria targeted molecules	MitoQ SkQ1 MTP-131 Hemigramicidin-TEMPO conjugates	(Smith and Murphy 2010) (Anisimov et al. 2008) (Chen et al. 2017) (Fink et al. 2007)
Bypassing respiratory complexes	Idebenone	(Haefeli et al. 2011, Erb et al. 2012)
Increasing mitochondrial numbers	Bezafibrate RTA-408	(Yatsuga and Suomalainen 2011) (Liu et al. 2016)
Modifying mitochondrial dynamics	P110	(Qi et al. 2012)

Table 4: Quinones and mitochondrial targeted molecules tested in clinical trials to treat mitochondrial dysfunction (Status May 2018).

Drug		Clinical trials			
Name	**Current status**	**Target disease**	**Identifier**	**Status**	**Sponsor**
Coenzyme Q_{10}	Dietary supplement	Progressive Supranuclear Palsy	NCT00328874	Completed	German Parkinson Study Group (GPS)
		Mitochondrial diseases	NCT00432744		University of Florida
		Dyslipidemia	NCT02407548		Sun Yat-sen University
Idebenone	Approved by EMA to treat LHON; Raxone®	LHON	NCT00747487 NCT01421381	Completed	Santhera Pharmaceuticals
			NCT02774005 NCT02771379	Ongoing	
		Friedreich's Ataxia	NCT00905268 NCT00993967 NCT00697073 NCT00537680	Completed	
			NCT00015808 NCT00078481 NCT00229632	Completed	National Institute of Neurological Disorders and Stroke
		MELAS	NCT00887562		Santhera Pharmaceuticals
KH176	In clinical development	Mitochondrial diseases	NCT02544217 NCT02909400	Completed	Khondrion BV
EPI-743	In clinical development	Friedreich's Ataxia	NCT01962363 NCT01728064	Completed	Edison Pharmaceuticals
		Rett syndrome	NCT01822249		
		Parkinson’s disease	NCT01923584		

		Mitochondrial and metabolic diseases	NCT01642056	Ongoing	
		Mitochondrial respiratory chain diseases	NCT01370447		
		Leigh syndrome	NCT02352896	Terminated	
		Pearson syndrome	NCT02104336		
A0001	In clinical development	Friedreich's Ataxia	NCT01035671	Completed	Penwest Pharmaceuticals Co.
EPI-589	In clinical development	Leigh syndrome	NCT01721733	Completed	Edison Pharmaceuticals
		Parkinson's disease	NCT02462603	Ongoing	
RP103	Approved in US to treat cystinosis; Procysbi®	Inherited mitochondrial disease	NCT02023866 NCT02473445	Completed	Horizon Pharma USA, Inc
MitoQ	In clinical development	Parkinson's disease	NCT00329056	Completed	Antipodean Pharmaceuticals, Inc.
		Chronic hepatitis C	NCT00433108		
		Multiple sclerosis	NCT03166800	Ongoing	Oregon Health and Science University
		Chronic kidney disease	NCT02364648		University of Delaware
		Aging	NCT02597023		University of Colorado, Boulder
		Non-alcoholic fatty liver disease	NCT01167088	Terminated	Antipodean Pharmaceuticals, Inc.
SkQ1	Approved in Russia to treat dry eye syndrome; Visomitin®	Mild-moderate dry eye syndrome	NCT02121301	Completed	Mitotech S.A
MTP131	In clinical development	Mitochondrial myopathy	NCT02367014	Completed	Stealth Bio Therapeutics Inc.
			NCT03323749	Ongoing	
		Primary mitochondrial disease	NCT02805790 NCT02976038		
		LHON	NCT02693119		

1.3.1 Coenzyme Q_{10}

Coenzyme Q_{10} (CoQ_{10}), and its species-specific analogues (CoQ_9 in rats) belong to a group of lipid soluble molecules present in lipid membranes of most mammalian cells. CoQ_{10} is the most common ubiquinone in humans, composed of a long isoprenoid chain attached to a benzoquinone head, that renders the molecule extremely hydrophobic (Figure 13). CoQ_{10} is also a pivotal component of the mitochondrial ETC and is present in the mitochondrial inner membrane. Upon reduction of the quinone (Q) by cellular reductases to its hydroquinone form (QH_2), this essential molecule helps to shuttle electrons from C-I and C-II to C-III of mitochondrial ETC. In its reduced form, CoQ_{10} also acts as an effective antioxidant by scavenging free radicals and hence preventing membrane lipid peroxidation.

Low CoQ_{10} levels are associated with many neurodegenerative and age-related disorders (Chhetri and Gueven 2016) and are also associated with reduced energy production and oxidative stress. A significant reduction of retinal CoQ_{10} levels has been described for many ocular diseases such as glaucoma, diabetic retinopathy, age-related macular degeneration and cataract formation and is reflected by significant increases in oxidative damage (Williams 2008, Qu et al. 2009, Chhetri and Gueven 2016). Pre-clinical studies hypothesised some beneficial effects of CoQ_{10} treatment in preventing premature cataractogenesis in human lens epithelial cells based on the ability of CoQ_{10} to prevent light-induced oxidative stress and cell death in human lens epithelial cell line, *in vitro* (Kernt et al. 2010). The antioxidant capacity of the reduced form of CoQ_{10} in mitochondria also protects cells from chemically induced apoptosis, depolarisation of mitochondria and caspase activation in pre-clinical studies (Alleva et al. 2001, Chen et al. 2011). CoQ_{10},

owing to its antioxidant properties, reportedly also protects against cardiac apoptosis in rats with ischemia-induced injury by upregulating Bcl2 gene expression (Khan et al. 2017). In a prospective, randomised, placebo-controlled study including 215 elderly individuals with cardiovascular diseases, supplementation with CoQ_{10} lowered lipid peroxidation of low-density lipoproteins which are known contribute to atherosclerosis (Alehagen et al. 2017, Thomas et al. 1997).

Although a number of pre-clinical studies indicate the efficacy of CoQ_{10} in treating various disorders, there are very few, well-controlled clinical trials to prove its efficacy in patients (Table 4). A randomised, placebo-controlled study conducted in patients with inherited mitochondrial cytopathies (Glover et al. 2010) and progressive supranuclear palsy (Stamelou et al. 2008) showed mild benefits of CoQ_{10} supplementation. Another phase 3 trial was conducted (trial concluded in 2017) to determine the efficacy of CoQ_{10} on patients with mitochondrial diseases, however, the results of this study have not been published so far. A meta-analysis conducted on randomised controlled trials of CoQ_{10} supplementation in healthy adults at risk of cardiovascular diseases suggests that there is insufficient evidence to draw any conclusions (Flowers et al. 2014). The overall lack of measurable therapeutic effectiveness of CoQ_{10} can likely be attributed to its very high hydrophobicity that is thought to interfere with drug absorption and distribution. To address this issue, several short-chain quinones are in development to treat mitochondrial diseases.

1.3.2 Idebenone

To address the major limitation of CoQ_{10}, its extreme hydrophobicity, newer short-chain quinone analogues have been developed aiming to increase their therapeutic potential by improving the bioavailability of the drug. One of those analogues, idebenone, was developed in the 1980's by Takeda Pharmaceuticals for the treatment of Alzheimer's disease. However, due to inconclusive trial results, idebenone was never approved in this indication (Thal et al. 2003).

Idebenone is characterised by a much shorter (10C when compared to >40C in CoQ_{10}), unbranched side-chain with a terminal hydroxyl group (Figure 13). When compared to CoQ_{10}, idebenone being significantly less lipophilic (logD of 3.9 versus 19.1) results in improved solubility of the molecule. This physicochemical difference affects its interaction with cellular enzymes, proteins and pathways and is believed to have an entirely different pharmacodynamic profile when compared to CoQ_{10} (Gueven et al. 2015). Similar to CoQ_{10}, idebenone can act as an electron carrier by carrying electrons from C-I and C-II to C-III in the mitochondrial ETC and act as an antioxidant. Since the vast majority of studies concentrated to assess these activities *in vitro* and not *in vivo*, the exact mode of action of idebenone remains unclear at present. It is thought that idebenone similar to CoQ_{10}, has to be bio-activated by cellular reductases from the Q to QH_2 form before it is able to act as an electron donor and antioxidant (Erb et al. 2012, Haefeli et al. 2011). In contrast to CoQ_{10}, idebenone is predominantly reduced in the cytoplasm by NQO1 (Erb et al. 2012, Giorgio et al. 2012, Haefeli et al. 2011) and not by the mitochondrial ETC. Many mitochondrial disorders are characterised by mitochondrial C-I dysfunction (Chhetri and Gueven 2016) and in this context it was

reported that the reduced form of idebenone can bypass C-I dysfunction by directly carrying electrons from the cytoplasm to C-III. This process was proposed to reactivate electron flow in the ETC to support ATP synthesis in the presence of dysfunctional C-I (Erb et al. 2012, Haefeli et al. 2011). Idebenone maintained membrane potential and ATP synthesis even in the presence of C-I inhibitor, rotenone (Giorgio et al. 2012). Independent of C-I, idebenone stimulates other metabolic pathways such as the glycerol-phosphate shuttle, which utilises NADH to generate high-energy electron equivalents for the mitochondrial ETC (Rauchová et al. 2006, Rauchová et al. 2011, Rauchová et al. 2012). Consistent with other quinones, idebenone also acts as a potent antioxidant that can prevent lipid peroxidation in rat myoblasts and human liver cells (Erb et al. 2012, Haefeli et al. 2011) and isolated brain mitochondria (Jaber and Polster 2015). Intraperitoneal administration of 200 mg/kg idebenone significantly reduced lipid peroxides in rats with pilocarpine-induced seizures (Ahmed 2014). However, at higher concentrations and in some specific cell lines, idebenone is reported to inhibit C-I and act as a pro-oxidant, which is an observation that remains poorly understood (Gueven et al. 2015, Yu-Wai-Man et al. 2017). One explanation for the reported pro-oxidative activity is that this observation was described for cell lines with extremely low levels such as NQO1, SHSY-5Y (Tai et al. 2011). While benzoquinones like idebenone need to undergo a two-electron reduction by cellular reductases like NQO1 to be activated, in the absence of NQO1, idebenone undergoes a one-electron reduction to the unstable semiquinone that results in elevated levels of oxidative stress and toxicity. However, the relevance of this one-electron reduction in a clinical scenario is unclear as idebenone-induced ROS damage or significant toxicity was never reported in patients.

Based on promising pre-clinical results (Buyse et al. 2008, Jauslin et al. 2002, Rustin et al. 1999), idebenone was used in clinical trials of various mitochondrial diseases (Table 4).

Idebenone was initially trialled in FRDA patients. In general, idebenone was well tolerated without any dose-limiting toxicity and improved neurological function in patients in a dose dependent manner (Di Prospero et al. 2007), but the improvement did not reach statistically significance (Lynch et al. 2010). Idebenone also reportedly stabilised overall neurological function and improved speech and fine motor skills in 68 paediatric patients with FRDA in an open label study (Meier et al. 2012). Although idebenone was also trialled in patients with MELAS (Table 4), the study results are not yet published, which suggest that no statistically significant effects were observed.

An initial, phase 2a trial conducted to assess the safety, efficacy and tolerability of idebenone in LHON patients showed minimal side effects even in high doses (Klopstock et al. 2011). Although best recovery of visual acuity was not statistically significant, there was a consistent trend of therapeutic benefit (Klopstock et al. 2011). Idebenone also protected from the loss of colour vision (Rudolph et al. 2013). These benefits from idebenone persisted despite the discontinuation of treatment for a median time of 30 months in a observational, prospective, cohort follow-up study (Klopstock et al. 2013). In these patients, a larger treatment effect was observed in patients to whom treatment was commenced less than 1 year after the onset of disease (Klopstock et al. 2013) suggesting a narrow window of opportunity.

Based on these data, idebenone was approved to treat visual impairment in LHON by the European Medial Agencies (EMA) in September 2015.

1.3.3 KH-176

Trolox, an antioxidant analogue of vitamin E (α-tocopherol), reduced ROS levels and normalised mitochondrial function in skin fibroblasts derived from patients with mitochondrial C-I deficiency (Koopman et al. 2008). Based on this observation with trolox, structural modifications were made to improve the efficacy, potency, chemical and metabolic stability of Trolox, which led to the development of the Trolox analogue KH176 by *Khondrion* (Figure 13). Although the developmental stages of the lead molecule are published, the characterisation of the physicochemical properties of KH176 are not detailed (Koopman et al. 2016). KH176 is reported to reduce ROS levels, normalise lipid peroxidation levels and significantly reduce the degeneration of retinal ganglion cells in a mitochondrial C-I deficient mouse model of Leigh disease, *Ndufs4*$^{-/-}$ (Haas et al. 2017). Although the detailed mode of action for this molecule is unclear, it is generally portrayed as an antioxidant. Based on the *in vitro* and *in vivo* evidence, KH176 was progressed to a phase-1 clinical trial in healthy volunteers (Table 4), where it was found to be safe and well tolerated with doses of up to 800 mg/day over 1 week (Koene et al. 2017). However, detailed pre-clinical efficacy data for KH176 are largely missing, which prevents speculation about its potential to alleviate symptoms of specific mitochondrial diseases.

1.3.4 EPI-743

EPI-743, α-tocotrienol quinone, is a *para*-benzoquinone analogue of Vitamin E that is also structurally closely related to KH176 (Figure 13) Tocotrienols are naturally occurring compounds isolated from plants and pre-clinical studies suggest that it possesses strong antioxidant, anti-inflammatory, anticancer, and neuroprotective properties (Packer et al. 2001, Serbinova et al. 1991). *Edison Pharmaceuticals* developed EPI-743 to treat inherited mitochondrial ETC diseases such as Leigh syndrome, LHON and FRDA (Shrader et al. 2011). EPI-743 is claimed to bind to cellular reductases like NQO1 and donate electrons in its reduced state, similar to other short-chain quinones; however, experimental evidence for this statement has not been published. With an EC_{50} of about 20 nM, EPI-743 is claimed to be significantly more potent as an antioxidant compared to CoQ_{10} or idebenone (Enns et al. 2012). EPI-743 is believed to protect from oxidative stress by increasing cellular levels of reduced glutathione by influencing the transcription of genes involved in glutathione homeostasis, which ultimately improves the cellular redox status (Pastore et al. 2013, Sadun et al. 2012). This hypothesis is supported by the results from a clinical trial, where after six months of treatment with EPI-743 significantly increased reduced glutathione levels in lymphocytes of Leigh syndrome patients were detected (Pastore et al. 2013).

Similar to idebenone, EPI-743 has intermediate oral bioavailability, which can be further increased by co-administration of food (Shrader et al. 2011). In a small, open-label study in 14 patients (13 children, 1 adult) with various mitochondrial diseases at risk of progressing to end-of-life care within 90 d, 12 patients survived, of which 11 patients showed clinical improvement and 10 patients reported improved quality of life. However,

partial relapse was seen in three patients (Enns et al. 2012). In another case report that included two siblings with LHON (m.14487T>C mutation), without any prior treatment, some spontaneous recovery in visual acuity was seen in both, which is common in patients with that mutation. However, after 7 weeks of treatment, the younger sibling showed a more rapid recovery of vision, where visual acuity improved from 20/50 to 20/20 OD; 20/20 OS when compared to the untreated, more severely affected older sibling with visual acuity of CF2 feet OD and 20/200 OS (Chicani et al. 2013). Including the patient from the above study, another small open-label trial with four additional LHON patients (3 with m.14484T>C, 1 with m.3460G>A mutation) was conducted. Reportedly, one mildly affected patient (with m.14484T>C, ND6 mutation) showed complete vision recovery while disease progression was arrested in the other 4 patients (Sadun et al. 2012).

EPI-743 was also trialled in Leigh syndrome patients in a small open-label study with 10 affected children (Table 4). Increased in the cellular levels of reduced glutathione were associated with improvements in the mitochondrial disease scale and motor function measures (Pastore et al. 2013). A drug closely related to EPI-743, A0001 (α-tocopheryl quinone), previously developed by *Edison Pharmaceuticals*, showed a dose-dependent improvement in Friedrich's ataxia rating scale (FARS) in a small, phase 2 double-blinded study trial that included 31 adults with FRDA (Lynch et al. 2012). A significant improvement in disease progression and neurological function after 400 mg t.i.d EPI-743 treatment for 2 years was reported in another phase 2 trial with 60 FRDA patients (Enns and Cohen 2017). However, this information was obtained from a review by Enns & Cohen, who gathered the information by personal communication from the responsible investigator, the complete results from this study are not published yet.

EPI-743 also improved motor function, decreased glutamine levels in CNS apart from combating oxidative stress in an open-label trail conducted on 10 patients with Parkinson's disease (Zesiewicz et al. 2016). Another phase-2, randomised, placebo-controlled clinical trial using EPI-743 to treat Rett syndrome, a progressive neurodevelopmental disorder is completed, however, results from this study are not yet published. A structural analogue of EPI-743, EPI-589 ((R)-troloxamine quinone), is currently tested in a phase 2, open label trial to treat Parkinson's disease (Trial identifier: NCT02462603).

Overall, since most trials using EPI-743 were conducted with very small patient numbers and mostly as open label trials, the effectiveness of EPI-743 to treat mitochondrial diseases is unclear at the moment.

1.3.5 RP-103

N-acetyl cysteine (NAC) is a well-known molecule that, similar to EPI-743, increases levels of the antioxidant, reduced glutathione (Atkuri et al. 2007, Moreira et al. 2007). RP103 (cysteamine bitartrate), which is structurally similar to NAC also increases glutathione levels (Figure 13). However, despite reducing oxidative stress levels in 27 patients with mitochondrial diseases, RP103 did not improve disease status or the quality of life (Mancuso et al. 2010). RP103 is also tested in a couple of open-label trials to test its safety and efficacy in children with mitochondrial diseases, however, the results of these studies are not published yet. Currently, there is no real evidence to support any therapeutic effects of RP103 in mitochondrial disease patients.

1.3.6 Mitoquinone

Traditionally, antioxidant molecules are known for their ability to combat oxidative stress in cells (Finkel and Holbrook 2000, Valko et al. 2007). To target drugs directly into the mitochondria aiming to improve their efficacy, the charged lipophilic triphenyl phosphonium (TPP^+) cation was conjugated to the hydroxyl moiety of idebenone resulting in mitoquinone mesylate (MitoQ, Figure 13) (Gruber et al. 2013, Murphy 2001). It is thought that the positive charge of TPP+ allows the molecule to enter the mitochondrial matrix in a membrane potential dependent manner (Murphy and Smith 2007). Similar to other short-chain quinones, like idebenone, MitoQ has to be bio-activated, i.e. reduced to its active quinol form in order to act as an antioxidant (James et al. 2005). This reduced form of MitoQ is described as an effective antioxidant that protects against lipid peroxidation and apoptosis induced by H_2O_2 (James et al. 2005, Lowes et al. 2008). Unlike other short-chain quinones such as idebenone and EPI-743, the bio-activation of MitoQ is entirely dependent on the activity of mitochondrial C-I due to its mitochondrial localisation. In the presence of rotenone, a C-I inhibitor, MitoQ was unable to prevent lipid peroxidation, which supports the hypothesis that MitoQ is activated exclusively by C-I of mitochondrial ETC (Kelso et al. 2001). Given that most mitochondrial disorders show a C-I deficiency, it questions the suitability of MitoQ for these disorders. Despite MitoQ being non-toxic to cells (*in vitro*) at concentrations of up to 10 µM (Kelso et al. 2001), it appears to increase oxidative stress by upregulating ROS generation in endothelial cells as well as in isolated mitochondria, which can lead to apoptosis (Doughan and Dikalov 2007). Furthermore, the charge dependent uptake of MitoQ into mitochondria harbours the risk to reduce the mitochondrial membrane potential further, which could be detrimental in some mitochondrial diseases.

Irrespectively, MitoQ was reported to improve survival in a mouse model of amyotrophic lateral sclerosis (ALS) along with a reduction in oxidative stress markers and neuronal loss in the spinal cord (Miquel et al. 2014). Beneficial, protective effects of MitoQ were also seen in the mouse models of Parkinson's and Alzheimer's disease (Smith and Murphy 2010). MitoQ also reportedly delayed disease progression by reducing neuronal cell loss apart from a significant reduction in inflammatory markers in a mouse model of multiple sclerosis (Mao et al. 2013). However, in another mouse model of inherited photoreceptor degeneration, MitoQ failed to restore mitochondrial C-I activity or glutathione levels. In fact, in this study, MitoQ increased the levels of reactive oxygen species (Vlachantoni et al. 2010). Despite the contradictory pre-clinical evidence, MitoQ was tested in a double-blind, placebo-controlled clinical trial with 128 newly diagnosed patients with Parkinson's diseases (Table 4). In this trial, MitoQ failed to influence disease progression compared to placebo (Snow et al. 2010). Although there is no evidence supporting the use of MitoQ use in mitochondrial disease patients, MitoQ is currently tested in patients with multiple sclerosis, age-related disorders, chronic kidney disease and non-alcoholic fatty liver disease (Table 4).

1.3.7 SkQ1

To improve the antioxidant capacity of the mitochondrial targeted quinones, similar to MitoQ, TPP^+ anion was conjugated to a plastoquinone to generate a compound known as SkQ1 (Figure 13). SkQ1 was reported to act as an antioxidant at sub-micromolar concentrations on isolated rat heart mitochondria (Skulachev et al. 2009). SkQ1 administered at 5 nmol/kg/day decreased intracellular ROS levels by two-fold in mice

and did not show any carcinogenic activity at that dosage (Skulachev et al. 2009). The reduced form of SkQ1, showed higher anti-oxidative and significantly lower pro-oxidative capacity when compared MitoQ (Skulachev et al. 2009). SkQ1 also prevented H_2O_2-induced apoptosis and was reported to prevent cardiolipin oxidation in the inner mitochondrial membrane (Skulachev et al. 2009). Without inducing liver cytochrome P450 enzymes, SkQ1 also reportedly prevented lipid peroxidation and prevented retinopathy and cataract formation in a OXYS rat model of accelerated ageing (Skulachev et al. 2009). The photoreceptor layer was also retained in these rats, which was lost in untreated animals (Gruber et al. 2013). This activity was supported by another study in OXYS rats, where SkQ1 increased behavioural activity and reduced mitochondrial damage without influencing mitochondrial numbers (Kolosova et al. 2017). It was hypothesised that due to its cytoprotective abilities, SkQ1 prolonged the life span of mice, crustacean and fungi at nM concentrations (Anisimov et al. 2008).

However, in another mouse model of polymicrobial sepsis, SkQ1 did not protect against reactive nitrogen species and did not improve mitochondrial autophagy. In contrast, in this study, SkQ1 exacerbated the mortality rate of the animals by almost 29 % (Rademann et al. 2017). SkQ1 also prevented dry eye syndrome induced by anaesthesia in rabbits, by suppressing oxidative stress and increasing glutathione levels. In these rabbits, SkQ1 decreased TNFα and IL6 levels and hence was described as an anti-inflammatory agent (Zernii et al. 2017). In another mouse study of drug induced acute inflammation, SkQ1 reduced the total number of inflammatory cells (Chelombitko et al. 2017). A randomised double-blind study of 240 patients reported the safety and efficacy of the use of SkQ1 in treating dry eye syndrome (Brzheskiy et al. 2015). However, in this study, the exact method of assessing the severity of dry eye by the investigator was not described in detail

and environmental factors that could conceivably influence dry eyes were not taken into consideration (Brzheskiy et al. 2015).

Based on these overall results, *Mitotec S.A.* developed SkQ1 as an eye drop formulation, marketed under the trade name Visomitin™ for treating dry eye syndrome, age-related macular degeneration and uveitis. Currently, Visomitin™ is only approved and marketed in Russia, while phase III trials are being planned in the US. Currently, there is limited evidence supporting the efficacy of SkQ1 in mitochondrial diseases.

1.3.8 MTP-131

An entirely different strategy to improve mitochondrial function involves the use of peptides instead of small molecules. Szeto-Schiller (SS) peptides selectively target and accumulate in the mitochondrial inner membrane by a membrane potential-independent manner (Szeto 2008). These peptides were initially developed as synthetic opioid receptor agonists to target the opioid receptor. SS peptides are characterised by four amino acids that include two basic amino acids that alternate with two aromatic amino acids (Zhao et al. 2003). MTP-131 was selected from a large family of peptides with this basic motive by optimising its protective and antioxidant activities. MTP-131 (Figure 13, also referred to as Bendavia, Ocluvia or Elamipreide) is described as a potent antioxidant tetrapeptide, that locates to the inner mitochondrial membrane with high affinity but by an unknown mechanism (Roestenberg et al. 2012). MTP-131 is reported to target the mitochondria-specific lipid cardiolipin in the IMM and prevents its peroxidation (Szeto 2008). This in-turn preserves the electron transfer function in the mitochondrial ETC and promotes OXPHOS and ATP synthesis (Birk et al. 2013).

MTP-131, with an *in vitro* IC_{50} in the nM range, scavenges H_2O_2, prevents lipid peroxidation and protects cell viability in a dose dependent manner (Szeto 2008). The inherent antioxidant properties of MTP-131 are believed to be due to the 2,6-dimethyl tyrosine (DMT) moiety located in its structure. This is supported by reports where the substitution of DMT eliminated the antioxidant properties of MTP-131 (Zhao et al. 2004). The antioxidant property of MTP-131 is believed to be responsible for *in vivo* protection against high-pressure induced cardiac failure (Dai et al. 2013) and ischemic renal injury (Szeto et al. 2011). MTP-131 also protected in a dose-dependent manner against chemically induced neurotoxicity in mice, increased ATP levels and prevented mitochondrial swelling at nM concentrations (Yang et al. 2009). In cultured retinal cells exposed to high-glucose levels, MTP-131 protected cell injury by attenuating ROS generation, stabilising membrane potential and decreasing the release of pro-apoptotic factors from the mitochondria (Li et al. 2011). MTP-131, in a rat model of drug-induced diabetes, protected the retinal structure apart from other diabetes-induced pathological changes (Huang et al. 2013). MTP-131, when administered by subcutaneous injection normalised visual acuity as well as diabetes in mice, whereas, when administered by eye drops, MTP-131 normalised only the visual acuity in diabetic mice (Alam et al. 2015). The antioxidant activity of MTP-131 also improved neurovascular coupling response, which is important in maintaining healthy cognitive function and also improved memory and motor skills in aged mice (Tarantini et al. 2018).

Based on these promising pre-clinical results, MTP-131 is currently tested in a number of clinical trials to treat mitochondrial and related disorders such as LHON, age-related macular degeneration, corneal disorders and mitochondrial myopathy (Table 4). Despite the availability of pre-clinical data, the results from these trials are required to substantiate the therapeutic potential of MTP-131 in mitochondrial disorders. However,

one major and obvious disadvantage of this approach is that peptides are not orally bioavailable and have to be injected systemically, which could hinder the development of this drug candidate for certain indications.

1.3.9 P-110

Under conditions of mitochondrial stress, hyper-activation of the protein Drp1 leads to the excessive mitochondrial fission that is associated with a number of neurodegenerative diseases (Reddy 2014). To treat excessive mitochondrial fission, a peptide inhibitor P110 that specifically targets the mitochondrial fission-related protein, Drp1 was recently developed by the pharmaceutical company, *Mitoconix Bio* (Qi et al. 2012). P110 was shown to inhibit mitochondrial and neuronal damage in an *in vitro* model of PD (Qi et al. 2012), reduced biomarkers of Huntington's disease (Guo et al. 2013) and reduced energy failure and oxidative stress while inhibiting mitochondrial fission in *in vitro* models of AD (Joshi et al. 2018) and ALS (Joshi et al. 2018). It is worth noting that these studies were conducted on cultured cells derived from patients with diseases with mitochondrial dysfunction, but they do not replicate the development of the chronic pathology as seen in humans. Intraperitoneal injection of P110 improved bioenergetics and decreased mitochondrial fission in a *in vivo* model of ischemic reperfusion injury and myocardial infarction (Disatnik et al. 2013). Although p110 may seem a promising strategy to treat neurodegenerative diseases, current evidence is still preliminary and additional research is needed.

1.3.10 Gene therapy

Gene therapy is the only curative approach that has been proposed to treat mitochondrial diseases caused by mtDNA and nDNA mutations that could correct the underlying defect in patients. For heteroplasmic mtDNA mutations, mitochondrial genome editing by delivering restriction endonucleases can be used to eliminate the mutant mtDNA sequences to re-populate the cells with wild-type mtDNA (Tanaka et al. 2002). Especially, TALENs and heterodimeric zinc finger nucleases have been used to target restriction endonucleases into the mitochondrion in a few proof-of-concept studies (Bacman et al. 2013, Gammage et al. 2014, Hashimoto et al. 2015). Mutations in mtDNA can also be compensated by allotropic expression. This strategy involves inserting a vector within the corresponding mitochondrial gene into the cells that is translated in the cytoplasm before the gene product has to be incorporated into the mitochondrial machinery. Intravitreal injection of an adeno-associated virus carrying the sequence for the C-I subunit ND4 was trialled in a small study on six LHON patients with the m.11778G>A mutation. This approach resulted in an enlargement of visual field but no other significant changes in outcome measures (Wan, Pei et al. 2016). For nDNA mutations, delivering the correct copy of the mutant gene by vectors such as the adeno-associated virus (AVV) has been trialled in a mouse model of LS and mitochondrial encephalomyopathy with varying degree of success (Meo et al. 2017, Torres-Torronteras et al. 2011). However, the main challenge in delivering gene products to the mitochondria is the hydrophobic nature of the specific proteins (Martin and Quigley 2004). Moreover, although it is assumed that the protein expressed by such genes integrates into the mitochondria and normalises its function, definitive proof is still missing.

Recently, a pluripotent stem cell model of primary CoQ_{10} deficiency that was caused due to CoQ_4 mutation was developed by CRISPR-Cas9 gene editing in pluripotent stem cells that could be used as a model for gene therapy (Romero-Moya et al. 2017). Based on these proof-of-concept studies, several clinical trials are also underway to trial this form of therapy (NCT02064569, NCT02161380). However, considering the significant number of gene defects (>350) associated with mitochondrial diseases (Rahman and Rahman 2018), developing gene therapies for every individual mutation and attracting investment for such single-dose therapies can be challenging.

1.3.11 Dietary modification

Mitochondria are at the heart of metabolic function of an organism to convert dietary nutrients into chemical energy. Consequently, dietary modifications have also been trialled to counteract pathology and disease severity of mitochondrial diseases. The most widely known and best studied of those is the ketogenic diet (KD).

KD predominantly consists of high fat (>90%), adequate amount of proteins, minerals, vitamins and very low carbohydrate content (Neal et al. 2008). In transgenic, AD mice (3 X TgAD), KD upregulated TCA cycle metabolites, lowered lipid peroxidation in the hippocampi (Pawlosky et al. 2017). KD also protected mice from sound-induced seizures, without affecting mitochondrial respiration when compared to a controlled-diet with intermittent fasting (Dolce et al. 2018). KD was also reported to alleviate seizure severity and decreased the percentage of damaged mitochondria which is believed to occur by upregulating mitochondrial Cyt C (Wang et al. 2018). In clinical trials, KD was reported to reduce body weight in overweight and obese adults, however, no significant changes

in cardiovascular risk factors were observed over up to two-year follow up period in a meta-analysis conducted on 19 randomised controlled trials (Brouns 2018, Naude et al. 2014). In contrast to the overweight but mostly healthy individuals, initiation of KD resulted in improved disease symptoms including consciousness and muscle strength in a 21-year-old patient with Leigh disease (Malojcic et al. 2004).

Despite good pre-clinical evidence that diverting energy generation away from carbohydrates to lipids can have beneficial effects in a potentially wide range of diseases that are associated with mitochondrial dysfunction (Brouns 2018, Gano et al. 2014, Lutas and Yellen 2013, Naude et al. 2014, Zhao et al. 2006) the exact molecular mechanisms for this effect are not clear. Therefore, at present, it remains unclear if and to what extent KD could be beneficial for which specific mitochondrial disease patients.

Vitamin K (VK), an essential nutrient, known for its role as a cofactor in blood coagulation (Suttie and Nelsestuen 1980). In the mitochondria, VK2 is reported to serve as an electron carrier and maintains ATP levels in fruit flies (Vos et al. 2012). Synthetic VK2 was also reported to prevent oxidative stress-induced cell death in vitro in cultured oligodendrocytes and neurons by blocking the accumulation of ROS and preventing cell death (Li et al. 2003, Sakaue et al. 2011). Supplementation of synthetic VK was reported to be beneficial in counteracting mitochondrial dysfunction-induced neurological disorders such as AD (Allison 2001) and to support neurocognitive functions (Ferland 2012).

Green leafy vegetables, certain plant oils such as canola, soybean and fish oil are the most common sources of dietary VK (Thane et al., 2006). The extracts of food rich in VK such as green leafy vegetables were reported for their antioxidant activity (Gupta et al., 2009) and their role in improving bone health. However, the exact role of VK from such dietary sources on mitochondrial function has not been reported yet.

1.3.12 Other therapeutic approaches to treat mitochondrial diseases

Several other therapeutic approaches have been tested or are underway in both pre-clinical as well as clinical trials to treat mitochondrial diseases (Table 5). Although these compounds are believed to show a down-stream effect of improving mitochondrial function, most of these compounds have multiple modes of action.

Resveratrol, that is known to promote biogenesis mediated by sirtunin (Sirt1, Sirt3 and Sirt5) activation (Menzies et al. 2013) is also known to act as an antioxidant, anticarcinogenic and neuroprotective agent (Albani et al. 2010, Athar et al. 2009, Gülçin 2010). Based on these pre-clinical results, resveratrol was progressed to a clinical trial in treating FRDA (NCT01339884). However, resveratrol failed to improve frataxin levels in peripheral blood mononuclear cells (Yiu et al. 2015). Other redox-active compounds such as curcumin and methylene blue have also been tested to treat mitochondrial disease, but with very little success (Jariyakosol et al. 2011). Anti-inflammatory molecules such as methyl prednisolone was also trialled in treating MS hypothesising that the anti-inflammatory response prevents relapse during acute phase of the disease (Sloka and Stefanelli 2005). However, the results from this trial is so far unavailable (NCT02424435). Clinical trials using antioxidants such as vitamin E and RG 2133 were terminated, suggesting none of them had any positive effects (Table 5). PPAR-γ agonist pioglitazone, that was reported to increase mitochondrial mass and provide neuroprotection was also trialled in treating mitochondrial diseases (Sauerbeck et al. 2011). However, the results from this study is also unavailable (Table 5). Compounds like cyclosporine that bind to mitochondrial permeability transition pore, control the influx of Ca^{2+} ions and mitochondrial swelling induced dysfunction also did not show any beneficial effects in an

open-label, non-randomised trial in 24 LHON patients (Leruez et al. 2018). Similarly, increased oxygen supply by using epoetin alfa also did not show any acute effects in patients with FRDA (Saccà et al. 2011).

Table 5: Additional therapeutic approaches to treat mitochondrial dysfunction (Status May 2018).

Drug	Clinical trials				
	Target disease	Identifier	Study type	Status	Sponsor
Resveratrol	FRDA	NCT01339884	Phase 1/2, interventional, non-randomised, open label, parallel assignment	Completed. End-point not met, no improvement in frataxin levels in peripheral blood mononuclear cells (Yiu et al. 2015).	Murdoch Children's Research Institute
Curcumin	LHON	NCT00528151	Phase 3, randomised, double-blind, placebo-controlled, parallel assignment	Completed. End-point not met, no data on visual acuity. However, antioxidant levels increased (Jariyakosol et al. 2011).	Mahidol University
Bezafibrate	Mitochondrial myopathy	NCT02398201	Phase 2, interventional, open label, single group assignment	Completed. End-point not met. However, LDL and triglyceride levels were decreased (Ørngreen et al. 2014).	Newcastle-upon-Tyne Hospitals
Epoetin alfa	FRDA	NCT00631202	Phase 2, interventional, non-randomised, open label, single group assignment, safety efficacy study	Completed. End-point not met, no improvement in frataxin levels. However, treatment well tolerated (Saccà et al. 2011).	Federico II University
Cyclosporine	Cardiac arrest	NCT01595958	Phase 3, interventional, randomised, parallel assignment	Completed.	Hospices Civils de Lyon

				Endpoint not met. Cyclosporine did not prevent second-eye involvement (Leruez et al. 2018).	
EGb761	FRDA	NCT00824512	Phase 2, interventional, randomised, double blind, placebo-controlled, parallel assignment	Completed, results unavailable	Ipsen
VP 20629	FRDA	NCT01898884	Phase 1, interventional, randomised, double blind, placebo-controlled, parallel assignment		Shire
Epicatechin	Muscular dystrophy	NCT03236662	Phase 2, open-label, single-group assignment		University of California
Methyl prednisolone	FRDA	NCT02424435	Phase 1, interventional, open label, single-group assignment		Children's Hospital of Philadelphia
RG 2133	Mitochondrial diseases	NCT00060515	Phase 1, open label, dose-escalation, safety, tolerability, PK, PD	Terminated	Repligen Corporation
Vitamin E	Peripheral neuropathy, MTPD	NCT00840112	Interventional, open label, single group assignment		Oregon Health and Science University
Varenicline	FRDA	NCT00803868	Phase 2/3, randomised, double-blind, parallel assignment		University of South Florida
RTA 408	FRDA	NCT02255435	Phase 2, randomised, double-blind, parallel assignment	Ongoing	Reata Pharmaceutical, Inc.
Arginine, citrulline	Mitochondrial diseases	NCT02809170	Interventional, open label, cross-over assignment		Tawam Hospital
Dichloroacetate	PDCD	NCT02616484	Phase 3, interventional, randomised, double-blind, cross-over assignment		University of Florida

Nicotinamide riboside	Mitochondrial diseases	NCT03432871	Interventional, open label, single-group assignment		Cambridge University Hospitals NHS Foundation Trust
TAK 831	FRDA	NCT03214588	Phase 2, interventional, randomised, double-blind, cross-over assignment		Takeda
Mitochondrial cocktail	Gulf war illness	NCT02804828	Interventional, placebo controlled	Unknown	University of California
Pioglitazone	Type II Diabetes	NCT01165190	Observational, cohort, prospective		Arizona State University

FRDA, Friedrich's Ataxia; MTPD, Mitochondrial Trifunctional Protein Deficiency; PDCD, Pyruvate Dehydrogenase Complex Deficiency

1.3.13 Summary

Overall, the results from a small number of clinical trials support the large amount of pre-clinical results that support the hypothesis that targeting mitochondrial function could be beneficial to treat diseases associated with mitochondrial dysfunction. However, there is only very limited evidence that supports the notion that the pre-clinical results can be easily translated to therapeutic outcomes in patients with mitochondrial diseases at the moment. Therefore, apart from a real need to develop additional drug candidates tailored to patients with specific mitochondrial diseases, this process has to be based on a detailed understanding around a molecular mode of action that is most likely to improve mitochondrial function or at least compensate for its loss.

Chapter 2: *In vitro* characterisation of novel short-chain quinones

2.1 Overview and rationale

Mitochondria are organelles that satisfy the energy demands of eukaryotic cells by synthesizing the majority of chemical energy in the form of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS) (Campbell and Reece 2005, Wallace et al. 2010). As a by-product, mitochondria also generate reactive oxygen species (ROS) under physiological conditions (Maurya et al. 2015, Zorov et al. 2014). Apart from energy generation and ROS production, mitochondria provide thermogenesis (Chouchani et al. 2016, Nedergaard et al. 2001), maintain cellular Ca^{2+} homeostasis (Ichas et al. 1994, Giorgio et al. 2017) and regulate cell death (Vakifahmetoglu-Norberg et al. 2017, Wojtczak and Zabłocki 2008). Tissues and cells that heavily depend on these functions, such as neurons (Leon et al. 2016) and muscle cells are therefore more sensitive to mitochondrial dysfunction (Zhang et al. 2016). Any physical or chemical insult or a genetic predisposition that leads to mitochondrial dysfunction can result in a range of disorders. These include true mitochondrial disorders such as Leigh syndrome (LS) (Legault et al. 2015), Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like Episodes (MELAS), Leber's hereditary optic neuropathy (LHON) (Gueven 2014) and Dominant Optic Atrophy (DOA) (Kushnareva et al. 2016). In addition, mitochondrial dysfunction is also present in a large number of neurological conditions such as Alzheimer's Disease (AD) (Lee et al. 2018), Parkinson Disease (PD) (Pickrell and Youle 2015) and Multiple Sclerosis (MS) (Dutta et al. 2006, Chhetri et al. 2018).

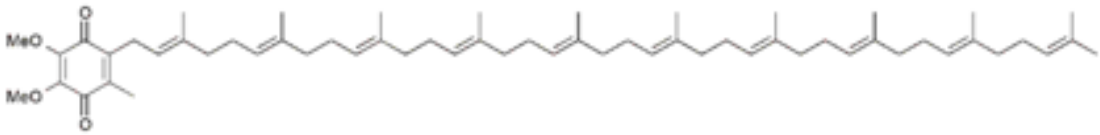
The chemical class of quinones is known for their antioxidant capacity apart from acting as signaling molecules and enzymatic co-factors (Ahmed 2014, Gueven et al. 2017, Smith and Murphy 2010, Stelmashook et al. 2015). Coenzyme Q_{10} (CoQ_{10}, Figure 14 i), a natural

ubiquinone that belongs to the class of benzoquinones is present in the lipid membranes of most cells and is a major component of the mitochondrial electron transport chain (ETC) (Wallace et al. 2010). CoQ_{10} in its reduced form, scavenges free radicals, acts as an effective antioxidant and prevents lipid peroxidation (Kernt et al. 2010). Due to its antioxidant ability along with lowered levels of CoQ_{10} in older individuals and patients with neurodegenerative disorders, CoQ_{10} has been widely proposed and tested as a therapeutic agent (Noh et al. 2013, Stamelou et al. 2008, Zhang et al. 2017). However, no significant improvement was observed upon the use of CoQ_{10} in clinical trials in patients with cardiovascular diseases (Flowers et al. 2014) (See section 1.3.1 for details). One of the main limitations of CoQ_{10} is its size combined with a very high lipophilicity (logD 19.12), which in turn affects its absorption and distribution and bioavailability (Gueven et al. 2015). Therefore, smaller variants of CoQ_{10} with higher solubility were developed.

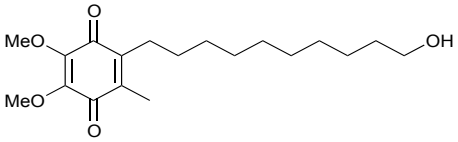
The short-chain benzoquinone idebenone (Figure 14 ii) was developed by Takeda Pharmaceuticals with the aim to retain the fundamental activities of CoQ_{10} in energy generation and as an antioxidant. This was approached by significantly improving the hydrophilicity of the compound. Takeda unsuccessfully tried to develop idebenone for the treatment of Alzheimer's disease (Miyamoto et al. 1999). Similar to CoQ_{10}, idebenone contains a benzoquinone moiety that is attached to a much shorter, less lipophilic tail with a terminal hydroxyl group to improve solubility (logD 3.91, compared to CoQ_{10} logD 19.12). Although structurally similar, it is now known that CoQ_{10} and idebenone significantly differ in their pharmacokinetics, bio-activation and modulation of cellular energy generation (Gueven et al. 2015). In the presence of physiological levels of CoQ_{10}, idebenone modulates mitochondrial energy generation, which suggests that the two molecules possess different functions (Haefeli et al. 2011). In fact, idebenone is an

efficient substrate for both C-II and C-III of the ETC when compared to CoQ_{10}. However, in contrast to CoQ_{10}, idebenone acts as a competitive inhibitor of C-I at higher concentrations (IC_{50}=5.9 µM), which is thought to be the consequence of a relatively slower off-rate at C-I compared to CoQ_{10} (Esposti et al. 1996, James et al. 2005, Sugiyama and Fujita 1985). Unlike CoQ_{10}, idebenone also engages in redox reactions outside of the mitochondria (Erb et al. 2012, Haefeli et al. 2011).

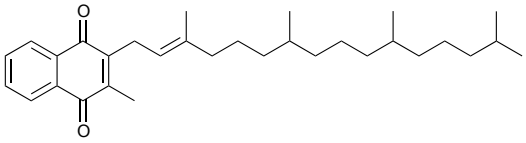
(i)



(ii)



(iii)



(iv)

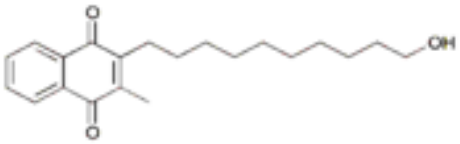


Figure 14: Molecular structures of (i) Coenzyme Q_{10}, (ii) Idebenone, (iii) Vitamin K1 and (iv) Novel short chain naphthoquinone (SCQ), #2

Idebenone is part of a larger class of so-called short-chain quinones (SCQs) that are described to show neuro- and mito-protective properties. In general, all of these compounds have been associated with different degrees of antioxidant activity and for some, a direct effect on cellular respiration has been reported (Erb et al. 2012, Geromel et al. 2002, Giorgio et al. 2012, Haefeli et al. 2011, Yu-Wai-Man et al. 2017). However, so far, no causative mode(s) of action has (have) been confirmed for this class of molecules that goes beyond pure associations. Xenobiotics are usually detoxified in the liver by enzymes such as the cytochrome P450 (CYP450) class of enzymes. However, CYP450 can reduce quinones by a one-electron reduction to the unstable semiquinone, which gives rise to superoxide. This quinone-induced superoxide production can damage cellular macromolecules and can lead to cell death (Erb et al. 2012, Ishihara et al. 2012, O'brien 1991). Therefore, most cells express cellular reductases like NAD(P)H quinone oxidoreductase-1 (NQO1) that competes with the CYP450 enzymes and catalyses a two-electron reduction to convert the quinone into its bioactive hydroquinone moiety (Haefeli et al. 2011). Hence, benzoquinones like idebenone would theoretically be safe only in the presence of NQO1. Unfortunately, some ethnic populations are associated with frequent NQO1 polymorphisms that result in either decreased or even complete absence of the enzyme activity (Hirai et al. 1998, Nebert et al. 2002, Hishida et al. 2005). Therefore, despite being effective antioxidants and protecting mitochondrial function, benzoquinones may not be effective in all individuals and in fact, they could cause ROS-induced toxicity. Apart from the dependence of idebenone on NQO1 for its bio-activation, clinical studies of idebenone revealed a very high first-pass metabolism (99 %) that significantly limits the amount of pharmacologically active drug in the plasma (Bodmer et al. 2009, Becker et al. 2010).

Apart from NQO1, quinones can also be reduced by additional member of the NQO gene family, NQO2 and NADH:CYP450 oxidoreductase. NQO2 is described to have substantially different substrate specificity and expression patterns. While NQO1 utilises NADH or NADPH as the electron donor, NQO2 prefers dihydro-nicotinamide riboside (Long and Jaiswal, 2000). Unlike NQO1, that is expressed ubiquitously, NQO2 is only expressed in selective tissues such as heart, kidney, lung, liver and skeletal muscle (Wu et al., 1997). However, the exact physiological role pf this enzyme has not been studied in detail. Idebenone and related short-chain quinones are shown to be excellent substrates for reduction by NQO1 (Haefeli et al., 2011; Erb et al., 2012), which therefore has been the focal point in this study.

Vitamin K (VK, Figure 14 iii), an essential nutrient, known for its role as a cofactor in blood coagulation (Suttie and Nelsestuen 1980). VK also plays a role during development and aging by modulating phosphorylation and regulating the activity of VK-dependent carboxylation of glutamate residues (Tsaioun 1999, Vermeer and Theuwissen 2011). Growing evidences suggest that VK2 exhibits anticancer activity in various cancer cell lines (Yoshida et al. 2003, Wei et al. 2010). This effect is thought to be mediated by blocking cell proliferation in the G1 phase of cell cycle (Tokita et al. 2006, Guerra et al. 2017). Some naturally occurring naphthoquinones like shikonin induce mitochondria-mediated apoptosis in human ovarian cancer cells, which supports its use as a chemotherapeutic in ovarian cancer (Shilnikova et al. 2018). Another study reported that injecting VK2 directly into the tumor-induced apoptosis of cancer cells in a ROS-mediated mechanism in a mouse model of human bladder cancer cells (Duan et al. 2016). However, the precise mechanism of action for this effect is still unknown. Synthetic VK analogues were developed to be selectively more toxic to cancerous cell lines (IC_{50} 0.24 – 3.83 μM) as opposed to non-cancerous cell line (IC_{50} 11.9 – 17.1 μM) (Prati et al. 2015).

On the other hand, VK is also reported to play a regulatory role in the maturing brain by upregulating the sulphatide turn-over in mouse brain (Sundaram and Lev 1990). In the mitochondria, VK is reported to serve as an electron carrier and maintains ATP levels in fruit flies (Vos et al. 2012). VK2 was also reported to prevent oxidative stress-induced cell death *in vitro* in cultured oligodendrocytes and neurons by blocking the accumulation of ROS and preventing cell death (Li et al. 2003, Sakaue et al. 2011). Bearing these diverse roles of VK in mind, coupled with VK deficiency in the ageing population, it is believed that the supplementation of VK may be beneficial to counteract mitochondrial dysfunction-induced neurological disorders such as AD (Allison 2001) and to support neurocognitive functions (Ferland 2012). For this purpose, synthetic VK analogues have been developed. Naphthoquinones such as VK (Figure 14 iii), are structurally closely related to benzoquinones (Figure 14 ii). However, in contrast to benzoquinones, they are bio-activated not only by NQO1, but also by additional enzymes such as Vitamin K oxido-reductase isoenzymes (VKORCS1, VKORL2). Based on this activation by multiple reductases, naphthoquinones should be at least theoretically in the absence of NQO1 show less ROS-induced toxicity compared to benzoquinones.

Synthetic 1,4-naphthoquinone derivatives substituted at the 2' and 3' position were shown to be protective against *in vitro* oxidative stress in a neuronal cells line (Josey et al. 2013). In fact, VK3 and its synthetically developed analogues increased the in vitro mitochondrial ATP turn-over and overall respiration in a mouse neuronal cell-line and reduced seizure activity in both zebrafish and mouse models (Chou et al. 2013, Rahn et al. 2014). These results suggest that energy production and mitochondrial function can be a good target to develop new naphthoquinone-based therapeutics to treat mitochondrial dysfunction.

Based on this reasoning, in the current study we synthesised and characterised 118 novel short-chain naphthoquinones (SCQs, Figure 14 iv,. See appendix 1 for full list of structures) to protect against mitochondrial dysfunction.

2.2 Aims and objectives

The precise protective mechanism of action of quinones is still unclear. The majority of the literature suggest that the ability to restore ATP levels and the antioxidant capacity of quinones is responsible for their mito-protective effects (Erb et al. 2012, James et al. 2005, Krylova et al. 2016, Skulachev et al. 2009, Suno and Nagaoka 1984). In this study, we aimed to generate effective SCQs that protect against mitochondrial dysfunction and work towards a better understanding of their molecular mode of action.

To achieve this aim, in this chapter, experiments were conducted to fulfill the following objectives:

- To characterise* 118 novel SCQs for their ability to protect against mitochondrial dysfunction and to protect cellular viability
- To evaluate the dependence of SCQs on NQO1 for their bioactivation and understand their relation to the cytoprotective ability of the SCQ
- To investigate the influence of SCQs on various parameters of cellular energy generation to improve our understanding of the mechanism(s) of action
- To work towards a structure activity relationship of our novel SCQs with regards to their cytoprotective activity in the presence of a mitochondrial toxin

*(*Note: All novel SCQs were synthesised by Dr. Krystel Woolley, Department of Chemistry, School of Natural Sciences, College of Sciences and Engineering, University of Tasmania, Hobart, Australia, as a part of her PhD thesis.)*

2.3 Methods

2.3.1 Cell culture

Regular maintenance of cells

The human hepatic carcinoma cell line, HepG2 was sourced from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). HepG2 cells were cultivated in regular growth medium composed of Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Castle Hill, Australia) containing 1 g/l glucose and 0.584 g/l glutamine, supplemented with 10 % heat inactivated fetal bovine serum (FBS, Gibco, Life Technologies, VIC, Australia) and 100 U/ml penicillin-streptomycin (Gibco, Life Technologies, VIC, Australia). Standard conditions for culturing cells were used i.e. 37° C, 5 % CO_2, 95 % humidity.

HepG2 cells were cultivated either in a T25 (25 cm^2) or T75 (75 cm^2) flask containing a total volume of 5 ml or 15 ml regular growth media respectively. For routine passaging of cells, HepG2 cells at about 80 % confluency, were first washed with 10 ml phosphate buffered saline (PBS, Sigma Aldrich, Castle Hill, Australia) to remove cellular wastes and media. Cells were then washed quickly with 1 ml ethylene diamine tetra-acetic acid (EDTA, 1 mg/ml) before adding 1 ml trypsin solution (Gibco, Life Technologies, VIC, Australia). Cells were allowed to detach from the flask by incubating them at 37° C for about 3-5 min in the incubator. The cells were checked under the microscope for detachment before trypsin was neutralised with 9 ml regular growth media. Cells were immediately resuspended to a single-cell suspension. The cells were then counted using

a haemocytometer and $2*10^6$ cells were seeded in a T25 flask in a total volume of 5 ml and placed in the incubator for future use.

Freezing and thawing cells

HepG2 cells were cultivated in T75 flask and harvested at 80 % confluency. Cells were detached from the flask using trypsin, followed by neutralising trypsin using regular growth media (see section 2.3.1) before collecting the suspension in a 10 ml centrifuge tube. The cell suspension was then pelleted using a bucket centrifuge (Elmi, Skyline Ltd, Latvia) at 500 g for 5 min. After removing the supernatant, the resultant cell pellet was resuspended in 1 ml of cryomedia (DMEM, 20 % FBS, 10 % dimethyl sulphoxide (DMSO, Sigma Aldrich, Castle Hill, Australia). The cell suspension (containing about $8\text{-}10*10^6$ cells / ml) was then transferred to a cryotube and cooled in a Mr. Frosty box containing isopropanol, to enable gradual cooling to -80° C at a constant rate of 1° C / min. After 24 h, the cryotubes were then transferred to liquid nitrogen for long-term storage.

To revive frozen cells, the cell suspension in a cryotube was rapidly thawed in a water-bath at 37° C until the pellet began to show first signs of melting. The ice-plug containing the still frozen cells was immediately transferred to a sterile, T75 flask containing 15 ml of pre-warmed regular growth media and placed in an incubator. The cells were allowed to adhere for 24 h before the culture media was replaced with fresh, regular growth medium to remove any residual DMSO and cell debris. Standard harvesting and sub-culturing of cells was performed after this point.

2.3.2 Preparing test compounds

All novel SCQs were synthesised by Dr. Krystel Woolley at the Department of Chemistry, School of Natural Sciences, College of Sciences and Engineering, University of Tasmania, Australia, while Santhera Pharmaceuticals (Pratteln, Switzerland) kindly donated idebenone. All compounds were dissolved in DMSO to prepare 10 mM stock solutions that were aliquoted as single-use-vials and stored at -20° C for future use. On the day of the experiment, 10 µM working drug solutions were prepared by diluting the 10 mM stock solution 1:1000 in growth media followed by intensive vortexing over 10 sec to ensure equal distribution. For all experiments, the final concentration of DMSO in the growth media was below 0.5 % v/v.

2.3.3 SCQs characterisation assays

Several *in vitro* assays were conducted to measure the effect of novel SCQs on different cellular, metabolic and mitochondrial parameters. Although 143 compounds were synthesised, some compounds could not be tested due to troubles while synthesizing them. Therefore, out of the 118 compounds tested (Complete list of compounds with structures in Appendix 1, Table A1-A9), up to the SCQ #98, the compounds had a naphthoquinone core, with different side chains, hence, were used in functional characterisation assays. Compounds #99 onwards, had a modified naphthoquinone core, and were therefore used in structural characterisation assays. Although all compounds were screened using the same assay, the results for compounds up to #98 were discussed in section 2.3.1, that shows the functional characterisation, while the subsequent

compounds are discussed in section 2.3.2, that reports the structural characterisation of the novel SCQs.

2.3.3.1 Protection of cell viability

Cytoprotection of HepG2 cells by the test compounds in the presence of the mitochondrial toxin, rotenone, was quantified as previously described with modifications (Heitz et al. 2012). Briefly, HepG2 cells were seeded in 96-well plates at 5000 cells/well in 100 µl regular growth medium. After overnight incubation under standard conditions, the cells were treated with test compounds at 10 µM in 100 µl fresh regular growth medium. After 2 days of pre-incubation, cells were challenged with 1 µM rotenone in the presence of 10 µM test compound in 100 µl Hank's balanced salt solution (HBSS, Life Technologies, Australia) for 6 h. This was followed by post-incubation for additional 18 h with only 10 µM test compound in 100 µl HBSS (Figure 15 i).

For ATP measurements, cells were washed with 100 µl PBS twice and then lysed in 40 µl lysis solution (4 mM EDTA, 0.2 % Triton X-100) for 5 min at room temperature (RT) on an orbital shaker at 200 rpm. Then, 10 µl of the lysate was mixed with 90 µl of 1:1 enzyme-substrate (E-S) mixture. This E-S mixture was composed of enzyme, luciferase solution (10 µg/ml firefly luciferase, 75 µM DL-dithiothreitol (DTT), 6.25 mM magnesium chloride ($MgCl_2$), 625 µM EDTA, 1 mg/ml bovine serum albumin (BSA) in 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.25) and the substrate, luciferin solution (600 µM D-luciferin, 75 µM DTT, 6.25 mM $MgCl_2$, 625 µM EDTA, 1 mg/ml BSA in 25 mM HEPES buffer, pH 7.25) in a white 96-well plate. Luminescence produced from the ATP-dependent oxidation of luciferin by the luciferase

enzyme (Figure 15 ii) was measured immediately using a Fluoroskan Ascent FL multi-mode plate-reader (Thermo scientific, VIC, Australia). Data is expressed as % of the untreated (no-rotenone) control. Data are presented as mean ± standard deviation (S.D.) from at least three independent experiments (n=3) with six replicate wells/experiment.

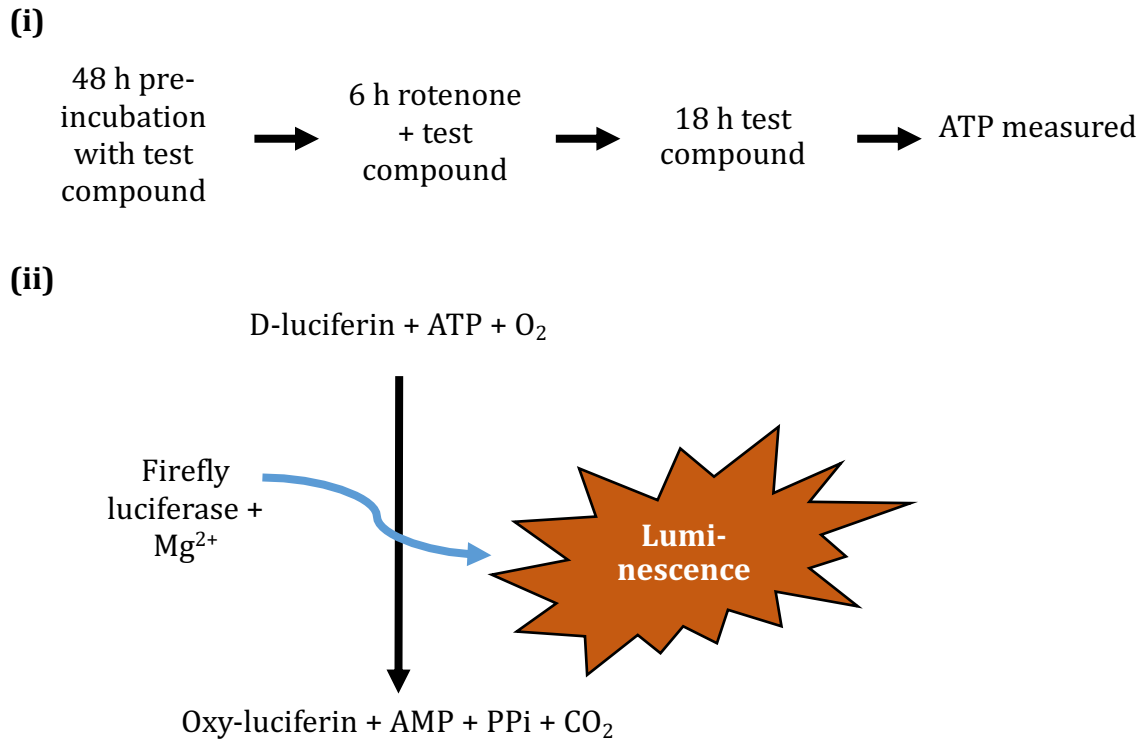


Figure 15: Assay for measuring cellular viability. (i) Schematic representation of the viability assay method and (ii) the chemical reaction involved.

ATP, adenosine triphosphate; AMP, adenosine monophosphate; PPi, inorganic phosphate

2.3.3.2 Acute rescue of ATP levels

In order to understand the extent to which novel SCQs are able to rescue ATP levels under conditions of mitochondrial dysfunction, mitochondrial C-I dependent respiration was selectively inhibited by rotenone. Since cells in culture typically generate energy by glycolysis, glucose-free media was used to reduce this effect as previously described (Haefeli et al. 2011). Therefore, the acute rescue of ATP levels by the test compounds was measured by co-treating HepG2 cells with SCQs (10 µM) and rotenone (10 µM) in glucose-free media (containing 2 % FBS). The resultant ATP levels were measured using the same luciferase/luciferin assay system as described under section 2.3.3.1.

Briefly, HepG2 cells were seeded in 96-well plates at 15,000 cells/well in 100 µl regular growth medium. After an overnight incubation under standard conditions, cells were treated with 10 µM rotenone in the presence of 10 µM test compounds in 100 µl of fresh glucose-free DMEM containing 2 % FBS for 1 h. For the measurement of ATP, cells were washed with 100 µl PBS twice and then lysed in 40 µl lysis solution (4 mM EDTA, 0.2 % Triton X-100) for 5 min at RT on an orbital shaker at 200 rpm. Then, 10 µl of the cell lysate was mixed with 90 µl of 1:1 E-S mixture in a white 96-well plate and luminescence produced from the ATP-dependent oxidation of luciferin by the enzyme luciferase was measured immediately using a Fluoroskan Ascent FL multimode plate-reader. Data is expressed as % viability compared to the untreated (no-rotenone) control. Data are presented as mean ± S.D. from at least three independent experiments (n=3) with six replicate wells/experiment.

2.3.3.3 NQO1 dependent reduction of quinones

Reduction of quinones by NQO1 was measured using a water-soluble tetrazolium dye (WST-1, 2-(4-iodophenly)-3-(4-nitrophenyl)-5-(2,4-disulphonly)-2H-tetrazolium, Santa Cruz Biotechnology, USA) as previously described with modifications (Haefeli et al. 2011, Tan and Berridge 2010). HepG2 cells were seeded at 10,000 cells/well in a 96-well plate in 100 µl of regular growth media. After 6 h of incubation, regular media was replaced with 100 µl fresh DMEM containing 0.3 g/L glucose and 2 % FBS and incubated further for 18 h. Later, media was replaced with 100 µl fresh DMEM containing 0.3 g/L glucose and 2 % FBS with/without quinones (10 µM) with/without dicoumarol (10 µM) and incubated for 1 h, before growth media was replaced with 100 µl HBSS with/without quinones (10 µM) containing WST-1 reagent (450 µM) without electron mediator (phenazine methosulphate, PMS) solution. Absorbance was measured at 450 nm every 2 min for 2 h at 37° C with a Multiskan GO spectrophotometer (Thermo Scientific, VIC, Australia). The difference between maximum and minimum absorbance in the absence of dicoumarol was defined as the total reduction of test compound by cellular reductases (Figure 16).

NQO1-dependent reduction of test compounds was defined as total reduction minus the difference between maximum and minimum absorbance in the presence of dicoumarol. The reduction by other reductases was defined as total reduction minus NQO1-dependent reduction of test compounds. Data are presented as mean ± S.D. of three independent experiments (n=3) with six replicate wells/experiment.

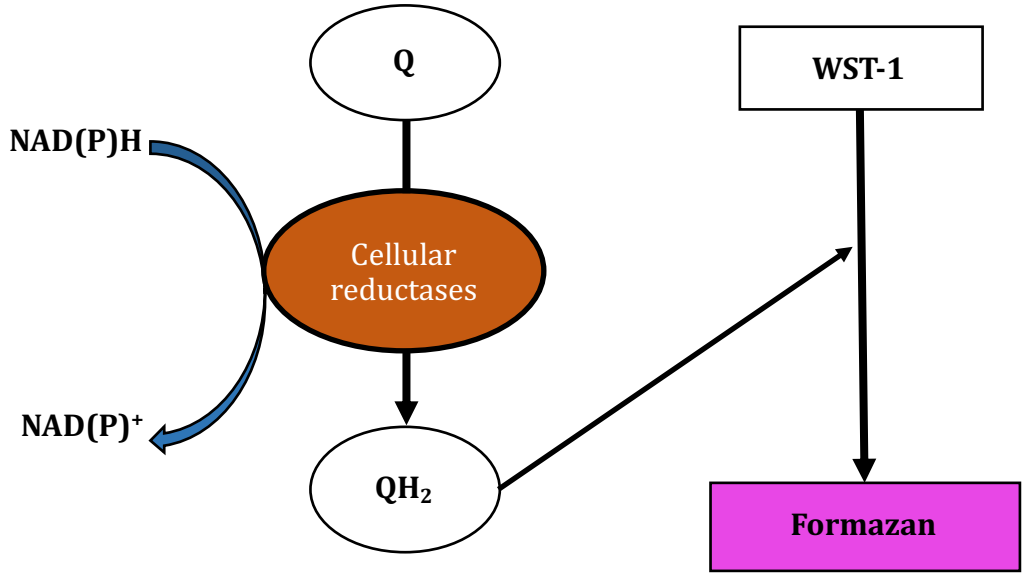


Figure 16: Schematic representation of the WST-1 assay for measuring reduction of quinones. Q, quinone; QH_2, hydroquinone; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; WST1, water soluble tetrazolium dye.

2.3.3.4 Accumulation of lactate in cell culture supernatant

Under normal conditions, cultured cells predominantly generate energy by glycolysis, which requires nicotinamide adenine dinucleotide (NAD^+) for conversion of glucose to pyruvate (Campbell and Reece 2005, Wallace et al. 2010). The NAD^+ required to maintain the glycolysis process is generated from the reduced NAD^+ (NADH) during the subsequent oxidation of pyruvate to lactate (Figure 17) (Vander et al. 2009). Therefore, the effect of SCQs on lactate accumulation in cellular supernatant was measured as previously described with modifications (Dykens et al. 2008, Haefeli et al. 2011). Briefly, HepG2 cells were seeded at a density of 150,000 cells/well in a 6-well plate in 1 ml regular growth media. After 24 h, the media was replaced with 1 ml challenge media (DMEM containing 25 mM glucose, 8 mM L-glutamine, sodium bicarbonate, pyridoxine HCl, 100 U/ml penicillin-streptomycin and 1 mM pyruvate) with/without test compounds at 10

µM final concentration. After 48 h, supernatant was collected for lactate measurement and the cells were lysed in lysis solution for protein measurement. For measuring lactate, 10 µl of supernatant was added to 90 µl of reaction buffer (10 mM potassium dihydrogen phosphate (KH_2PO_4) pH 7.8, 1 mg/ml BSA, 0.5 mM phenazine methosulphate (PMS), 2 mM EDTA, 0.3 mM dichlorophenolindophenol (DCPIP), 0.08 mM NAD^+, 0.5 U/ml glutamate-pyruvate-transaminase (GPT), 1.5 mM glutamate, 1.25 U/ml lactate dehydrogenase, LDH) in a 96-well plate (Figure 17). Absorbance was measured using Multiskan GO spectrophotometer (Thermo Scientific, VIC, Australia) at 600 nm every 2 min over a period of 60 min, at 30° C. Data is calculated as Lactate/Protein compared to the untreated (no-quinone) control. Since the lactate levels were measure as mM and the protein as mg/ml, the overall data is presented as lactate levels (µmol/mg protein), mean ± S.D. of three independent experiments (n=3) with three replicate wells/experiment.

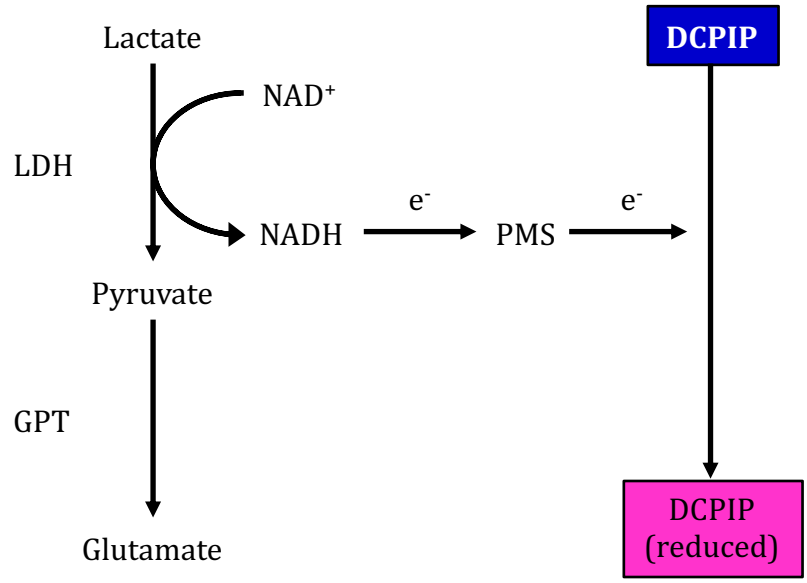


Figure 17: Schematic representation of the lactate assay.

LDH, lactate dehydrogenase; GPT, glutamate pyruvate transaminase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; PMS, phenazine methosulphate; DCPIP, dichlorophenolindophenol.

2.3.3.5 Accumulation of β-hydroxy butyrate in cell culture supernatant

The effect of quinones on fatty acid metabolism was measured by quantifying β-hydroxy butyrate (BHB), a by-product of fatty acid metabolism, in the cell culture media. Briefly, HepG2 cells were seeded at 10,000 cells/well in 100 µl regular growth media in a 96-well plate. After 24 h incubation, media was replaced with 100 µl fresh media containing test compounds (10 µM) and incubated for further 72 h. After incubation, supernatant was collected for quantifying BHB levels. To 50 µl of reaction mixture (containing 0.5 mM PMS, 2.5 mM NAD^+, 0.00625 U BHB-dehydrogenase (BHB-dh), 10 µM DCPIP in 100 mM Trizma hydrochloride (Tris HCl) buffer, pH 7.8), 50 µl of cell supernatant was added to initiate the reaction (Figure 18). Absorbance was measured at 600 nm every 30 sec for 10 min at RT using a Multiskan GO spectrophotometer (Thermo Scientific, VIC, Australia). A standard curve was plotted with known concentrations of BHB to estimate the BHB levels in the supernatants of test compounds-treated cells. Data represents the mean ± S.D. of three independent experiments (n=3) with six replicate wells each.

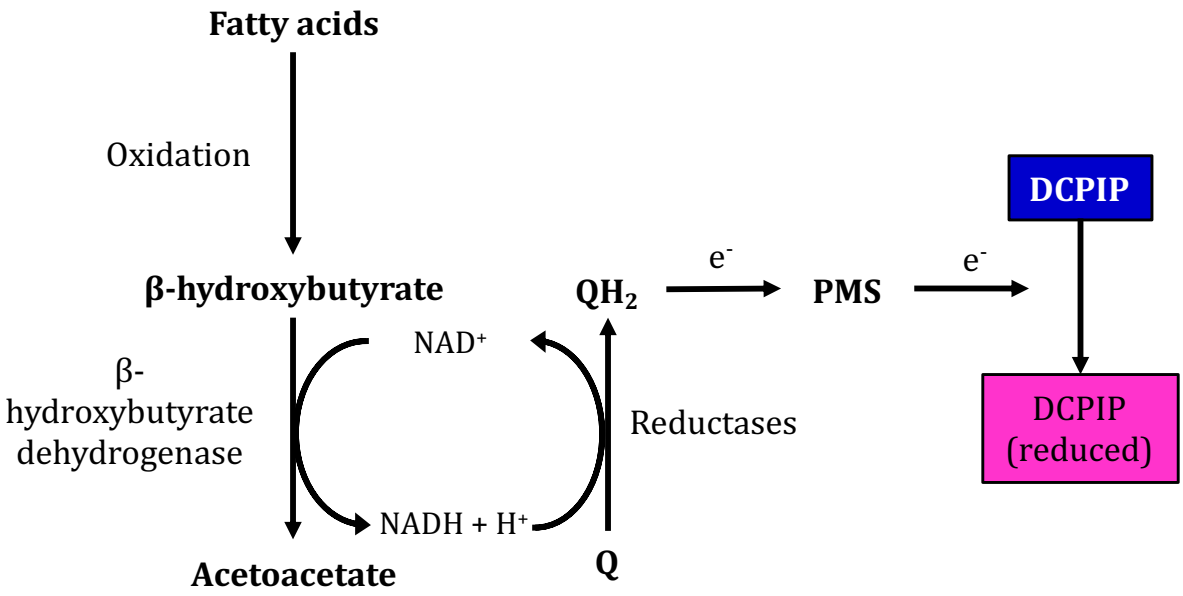


Figure 18: Schematic representation of the beta-hydroxybutyrate measurement assay.

NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; Q, quinone; QH_2, hydroquinone; PMS, phenazine methosulphate; DCPIP, dichlorophenolindophenol.

2.3.3.6 Mitochondrial mass

The mitochondrial mass of normal cells is continuously changing during growth and differentiation of cells. Under conditions of mitochondrial dysfunction, cells can upregulate mitochondrial mass as a compensatory mechanism to keep up with the cellular metabolic and ATP demands (Lee et al. 2002, Noh et al. 2013). Therefore, the influence of novel SCQs on mitochondrial mass was measured as previously described (Erb et al. 2012). Mitochondrial mass was measured using the fluorescent dye MitoTracker Green (Life Technologies Australia Pty Ltd, VIC, Australia). Briefly, HepG2 cells were seeded in 96-well plates at 20,000 cells/well in 100 µl regular growth media. After 6 h incubation under standard conditions, cells were treated with 10 µM test compounds in 100 µl regular growth medium for 72 h. For the measurement, cells were

washed with 100 µL HBSS per well once before incubating for 30 min under standard conditions with 400 nM MitoTracker Green (Life Technologies Australia Pty Ltd, VIC, Australia) in 100 µL HBSS per well. Subsequently, cells were washed twice with 50 µL PBS per well and then fluorescence was quantified immediately in 50 µL PBS per well (485 nm excitation and 538 nm emission) using a Fluoroskan Ascent FL multimode plate-reader (Thermo scientific, VIC, Australia). Data is expressed as % mitochondrial mass compared to the untreated (no-test compound) control. Data represents the mean ± S.D. of three independent experiments (n=3) with six replicate wells each.

2.3.3.7 Oxidative stress

To quantify the effect of quinones on levels of basal lipid peroxidation, HepG2 cells were seeded at a density of 20,000 cells/well in a black 96-well plates in 100 µL regular growth medium. After 24 h, cells were loaded with 10 µM BODIPY 581/591 C11 (Thermo Fisher Scientific, VIC, Australia) in 100 µL of fresh HBSS per well for 30 min. Subsequently, excess dye was removed by washing once with HBSS once before the cells were incubated with/without test compounds (10 µM) and incubated for 1 h. Cells were then washed three times with 100 µl PBS before fluorescence was quantified (Ex/Em 490/520 and Ex/Em 490/600) in 50 µl PBS using Fluoroskan Ascent FL multimode plate-reader (Thermo scientific, VIC, Australia). The ratio of red to green fluorescence was calculated as described by the manufacturer and presented as % of untreated (no-test compound) control cells. Data represents the mean ± S.D. of three independent experiments (n=3) with six replicate wells each.

2.3.4 Protein quantification

Protein content per well was used to standardise the individual values of the 96 well assays described above. For this purpose, HepG2 cells were first washed with 100 µl/well PBS and lysed using 40 µL lysis solution (see section 2.3.3.1 for details). The DC protein assay kit (Bio-Rad laboratories Pty Ltd, NSW, Australia) was used according to manufacturer's instructions. Briefly, 10 µl of cell lysate or protein standards, 0-2 mg/ml BSA (Sigma Aldrich, Castle Hill, Australia) were added in triplicate to a transparent 96-well plate. Reagent A' was prepared by mixing 20 µl of reagent S (surfactant) with 1 ml of reagent A (alkaline copper tartrate). To each well containing cell lysate/protein standard, 25 µl of reagent A' and subsequently 200 µl of reagent B (folin's agent) was added. The 96-well plate was kept on an orbital shaker at 200 rpm for 5 min followed by incubation at RT for another 10 min. The resulting absorbance at 750 nm was measured using a Multiskan GO spectrophotometer. Protein levels in cell lysate were calculated using the protein standard curve plotted from the absorbance derived from known BSA standards as described by the manufacturer.

2.3.5 Statistical analysis

All data are represented as mean ± S.D. as denoted in the figure legends. Statistical analysis to measure the significance between different groups was performed using Student *t*-test using GraphPad prism software (GraphPad Software Inc., CA, USA).

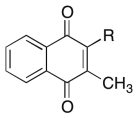
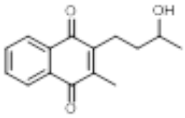
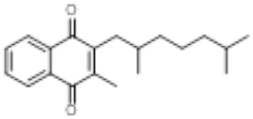
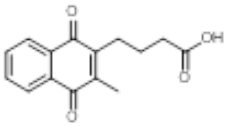
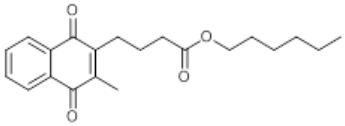
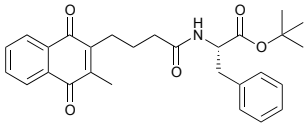
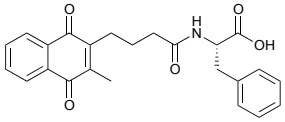
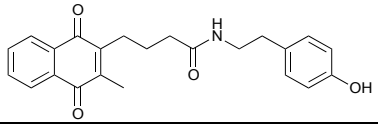
2.4 Results

2.4.1 Functional characterisation of novel SCQ's

The following assays were designed to understand the effect of SCQs on cellular and metabolic parameters that can be affected as a consequence of mitochondrial dysfunction. The novel SCQs up to compound #98 were generated by changing the functional group attached to the side chain, while maintaining the same naphthoquinone core. To analyse their biological effects, they were divided based on the terminal functional groups into slightly-polar (A), aliphatic (B), acids (C), aliphatic esters (D), amino esters (E), amino acids (F) and amino alcohols (G) (see representative example structures in Table 6, complete list of compounds and structures in Appendix 1, Table A1-A7).

In the current section, only the results for SCQs up to #98 are discussed. Compounds from SCQ #113 onwards, displayed a modified naphthoquinone core and were used for structural characterisation assays; the results of which are discussed in section 2.4.3.

Table 6: Structures of representative novel short-chain quinones classified based on the functional group attached on the side chain

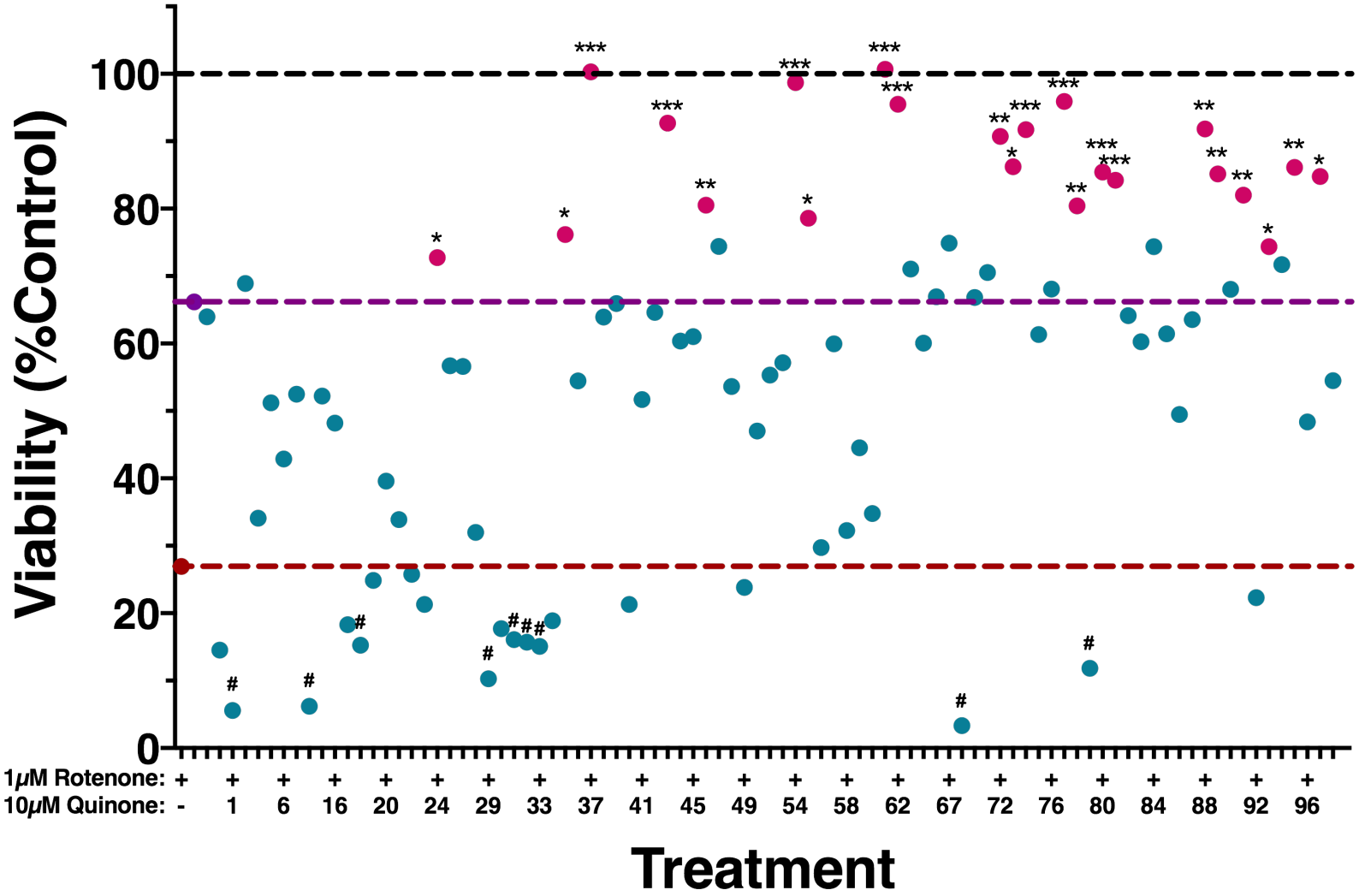
Category	Compound number	Structure
Novel SCQ skeleton	N/A	
(A) Slightly polar	#24	
(B) Aliphatic	#26	
(C) Acid	#23	
(D) Aliphatic ester	#34	
(E) Amino ester	#36	
(F) Amino acid	#37	
(G) Amino alcohol	#74	

2.4.1.1 Cytoprotective effects of novel SCQs under conditions of mitochondrial dysfunction

As an initial screen for the novel SCQs, their effect on cellular viability in the presence of rotenone-induced mitochondrial dysfunction was measured (Figure 15). All SCQs were initially screened at 10 µM, comparable to previously published studies that used similar compounds (Erb et al. 2012, Yu-Wai-Man et al. 2017). Over a total period of five days, rotenone (1 µM) decreased cellular viability to 26.9 ± 7.9 % compared to the sham-treated cells (red-dotted line, Figure 19 i). The presence of idebenone (Id, 10 µM) protected cell viability up to 66.2 ± 12.0 % of the sham-treated cells (purple-dotted line, Figure 19 i). Strikingly, out of the screened 87 novel SCQs, more than 20 compounds improved cell viability significantly better than idebenone (pink data points, Figure 19 i). While more than half of the compounds protected cell viability to levels above rotenone-treated cells, nine SCQs were cytotoxic at 10 µM and reduced cell viability further than rotenone alone (#, Figure 19 i).

When SCQs were grouped according to their functional groups, all groups except B and D significantly increased cell viability when compared to rotenone-treated (no SCQ) cells (Figure 19 ii). Compounds in group A protected cell viability up to 42.6 ± 23.7 %, while group C to 49.9 ± 21.8 %, group E 64.8 ± 8.2 %, group F to 69.7 ± 25.6 % and group G to 76.5 ± 15.9 % (Figure 19 ii). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

(i)


Viability (%Control)
100
80
60
40
20
0
1μM Rotenone: +
10μM Quinone: - 1 6 16 20 24 29 33 37 41 45 49 54 58 62 67 72 76 80 84 88 92 96
Treatment

(ii)

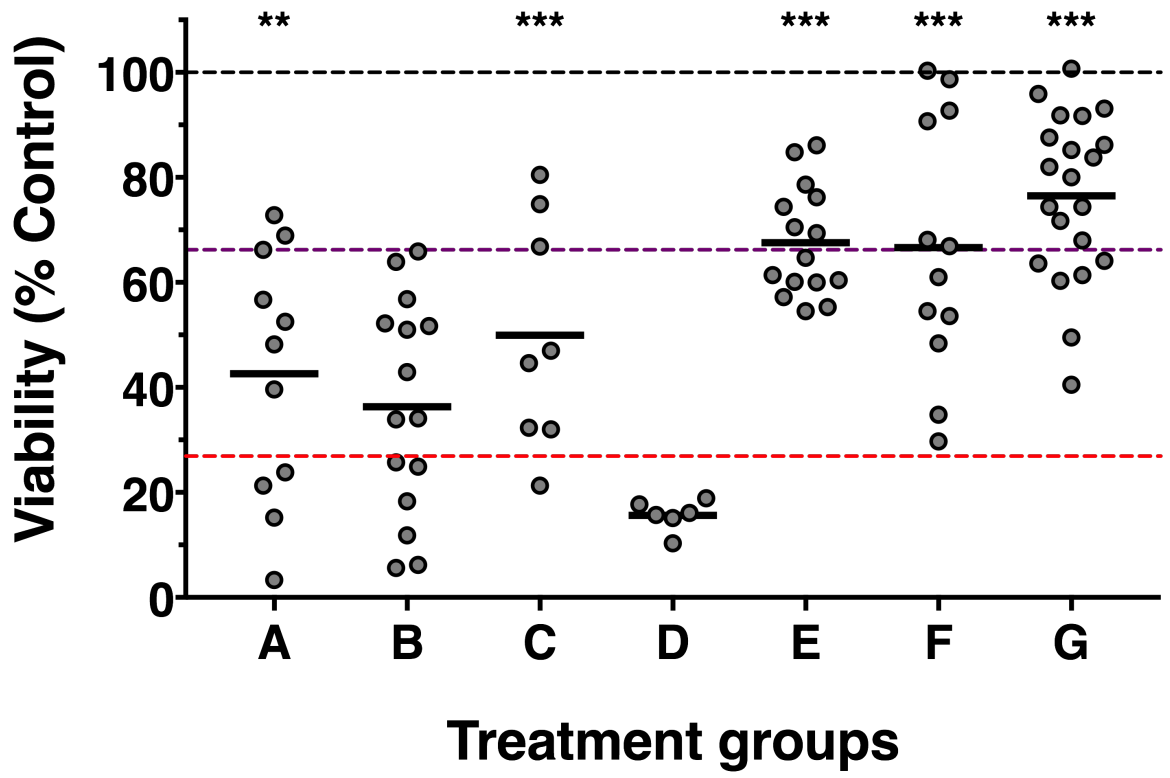
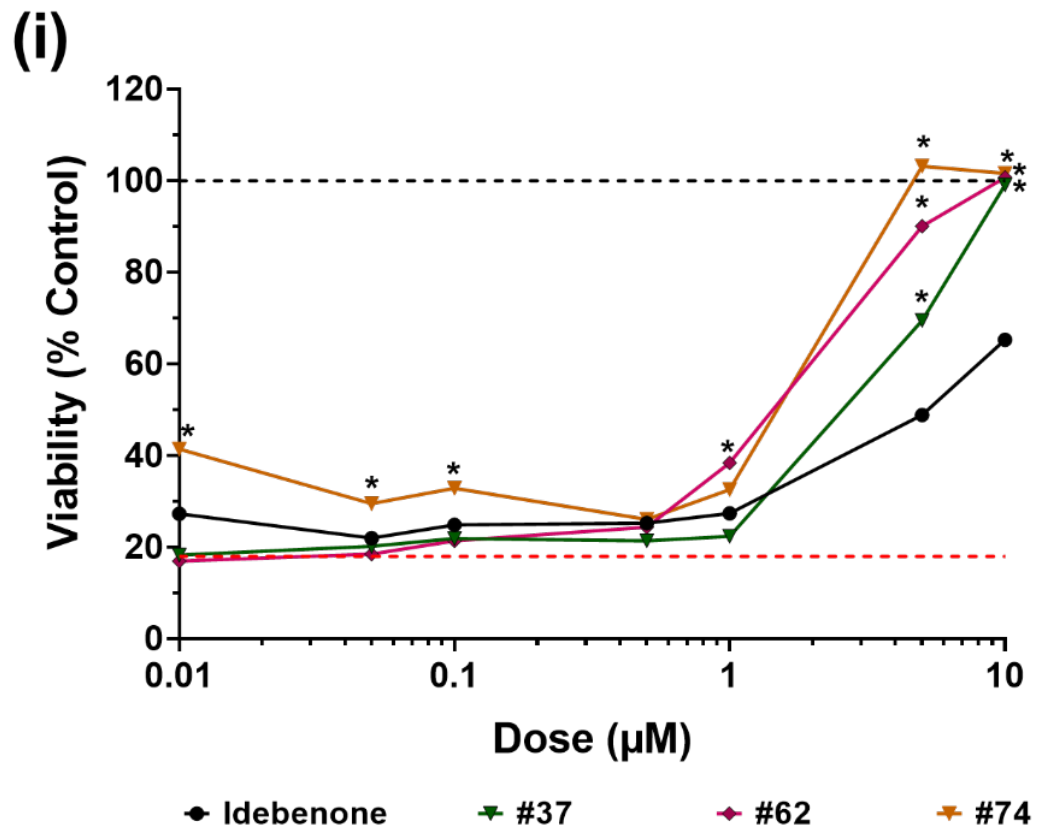
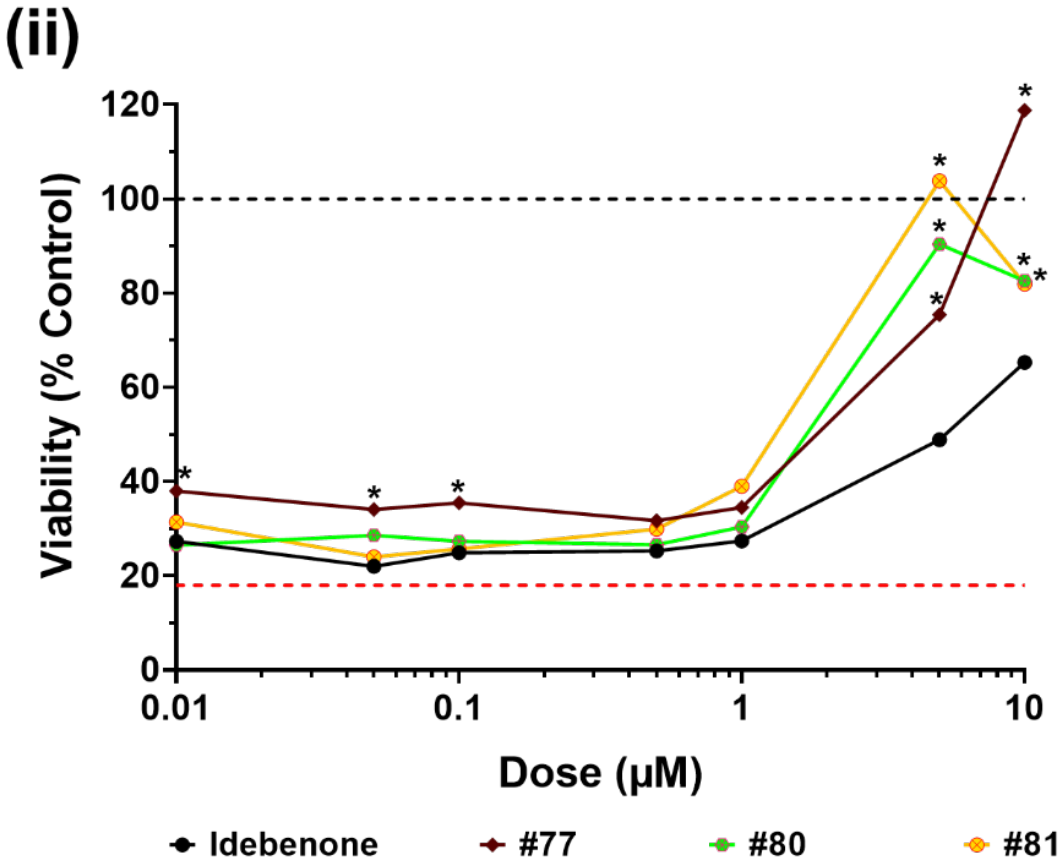


Figure 19: Cytoprotection by novel short-chain naphthoquinones (SCQs) in the presence of rotenone. Cytoprotection by test compounds (10 µM) in the presence of rotenone (1 µM) was expressed as percentage of sham-treated (no rotenone or SCQ) HepG2 cells. Black, red and purple dotted lines represent the viability of sham, rotenone and idebenone (Id, 10 µM)-treated cells, respectively. **(i)** Pink data points represent the SCQs that improve viability significantly better than Id. Significance was calculated by using Student *t*-test (p^* <0.05, p^{**}<0.01, p^{***}<0.001 cytoprotection by SCQs against Id-treated cells, $p\#$<0.05 cytoprotection by SCQs against rotenone-only treated cells) **(ii)** Cytoprotection by test compounds categorised according to their functional groups (p^* <0.05, p^{**}<0.01, p^{***}<0.001 cytoprotection by SCQs against rotenone-only treated cells).
Significance was calculated by using Student *t*-test.
Each data point represents one SCQ, data is expressed as mean ± S.D. of at least three independent experiments (n=3) with six replicates within each experiment. Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.
Mn, Menadione; VK, Vitamin K; A, Slight polarity; B, Aliphatic; C, Acid; D, Aliphatic ester; E, Amino ester; F, Amino acid; G, Amino alcohol.

2.4.1.2 Dose dependency

Some SCQs like #37, #61, #62, #77 (10 μM) restored viability of HepG2 cells in the presence of rotenone (1 μM) to almost 100 %, when compared to sham-treated cells (Figure 19). It was therefore of interest to measure how effective these SCQs would be at lower doses. For this purpose, cell viability was measured by using SCQ concentrations ranging from 0.01 μM to 10 μM. Six out of the nine tested SCQs including #37, #62, #74, #77, #80 and #81 showed significantly better cytoprotective activity at 5 μM compared to idebenone (Figure 20 i, ii). In fact, #62 also showed significantly better cytoprotection than idebenone at 1 μM (Figure 20 i). Compared to idebenone, compounds #74 and #77 showed significantly better cytoprotection at 0.01, 0.05 and 0.1 μM (Figure 20 ii). However, three of the nine tested SCQs (#72, #73 and #91) did not protect viability better than idebenone at 5 μM or less (Figure 20 iii).


(i)
Viability (% Control)
120
100
80
60
40
20
0
0.01
0.1
1
10
Dose (µM)
Idebenone
#37
#62
#74


(ii)
Viability (% Control)
120
100
80
60
40
20
0
0.01
0.1
1
10
Dose (µM)
Idebenone
#77
#80
#81

1

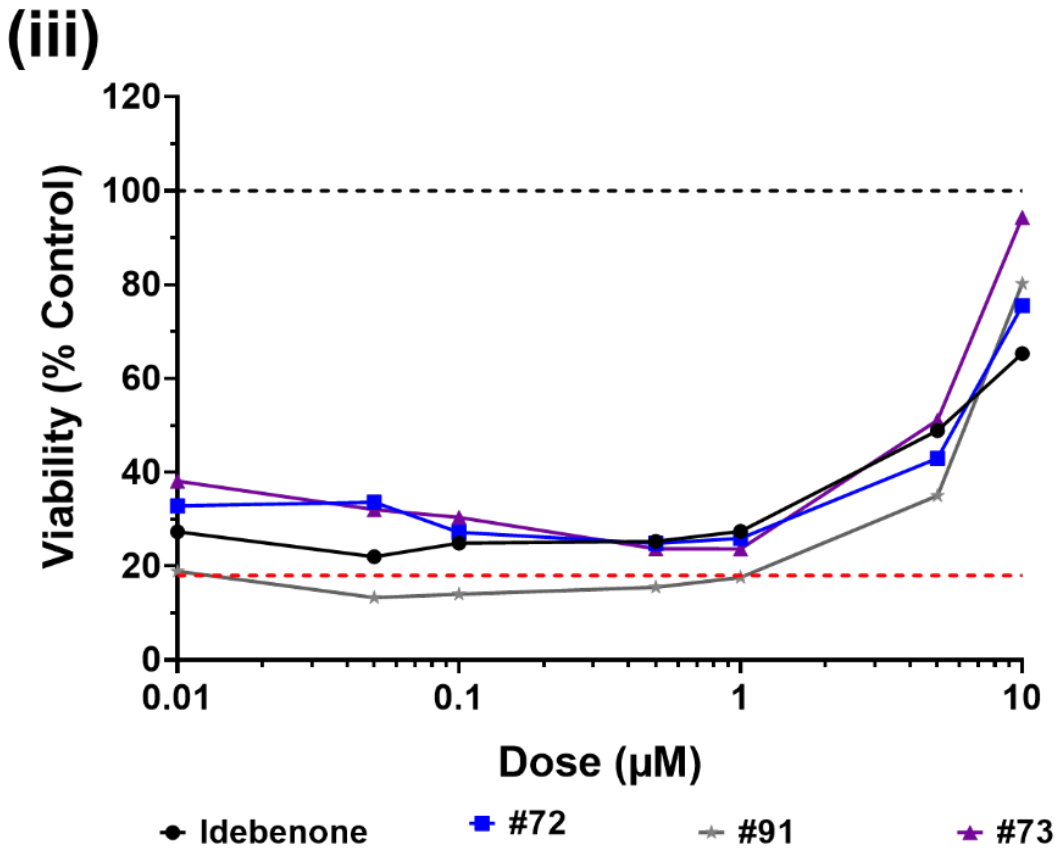


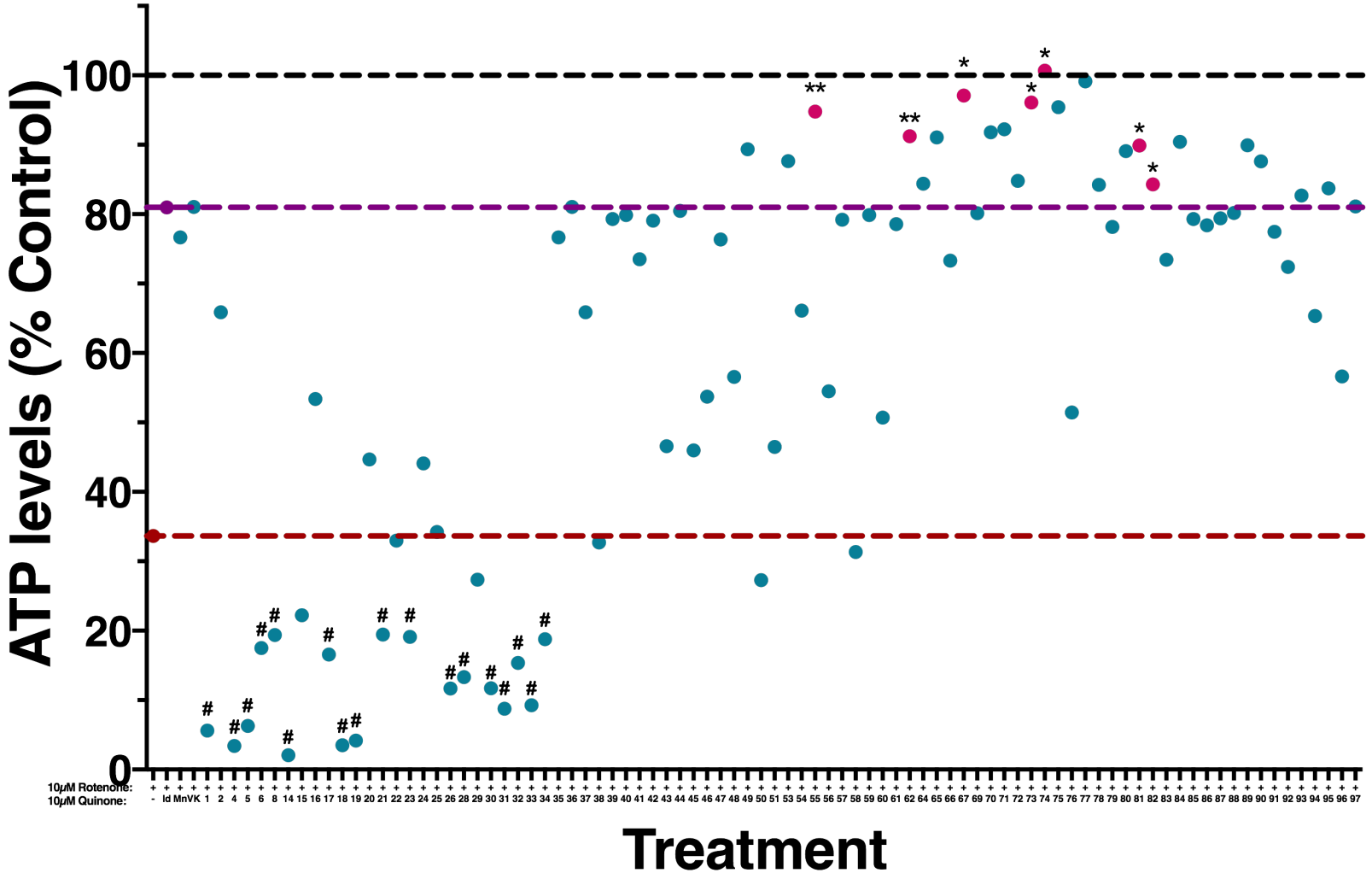
Figure 20: Dose dependency of short-chain naphthoquinones (SCQs) for protecting cell viability. Cytoprotection by selected SCQs, (i) Idebenone (Id), #37, #62, #74, (ii) Id, #77, #80, #81 and (iii) Id, #72, #91 and #74 at 0.01 µM to 10 µM, in the presence of rotenone (1 µM) was measured and expressed as percentage of sham-treated HepG2 cells. Each coloured, solid line represents one SCQ, black and red dotted line represent sham- and rotenone-treated cells. Data from one representative out of three experiments (n=3) is presented and is expressed as mean of six replicates. Significance was calculated by using Student *t*-test to compare cytoprotection by SCQs against Id treated cells ($p^* < 0.05$). Error bars were omitted for clarity.

2.4.1.3 Acute rescue of ATP levels by novel SCQs in the presence of high-dose rotenone

One of the first and dominant parameters affected by mitochondrial dysfunction is typically cellular ATP synthesis. Hence, after assessing cytoprotection by the novel SCQs, acute rescue of ATP levels in the presence of drug-induced (rotenone, 10 µM) mitochondrial dysfunction was measured as previously described for a panel of benzoquinones (Erb et al. 2012). A high dose of rotenone (10 µM), acutely reduced cellular ATP levels down to 33.6 ± 11.1 % compared to sham-treated cells (red-dotted line, Figure 21 i) within 1 h. Under these conditions, the presence of idebenone (Id, 10 µM) rescued cellular ATP levels to 81 ± 9.4 % of the sham-treated cells (purple-dotted line, Figure 21 i). Out of the 87 screened novel SCQs (10 µM), seven compounds rescued ATP levels significantly better than idebenone (pink data points, Figure 21 i) and more than half of the compounds rescued ATP to levels above rotenone-treated cells. However, for 18 SCQs, the combination of SCQ and rotenone reduced ATP levels significantly lower than rotenone treated (no SCQ) cells (Figure 21 i).

When SCQs were grouped according to their functional groups, all groups except B and D significantly increased ATP levels when compared to rotenone-treated (no SCQ) cells (Group A: 49.2 ± 30.9 %, Group C: 51.7 ± 33.8 %, Group E: 81.9 ± 12.6 %, Group F: 59.4 ± 12.6 % and Group G: 84.3 ± 9.0 %) (Figure 21 ii). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

(i)


ATP levels (% Control)
100
80
60
40
20
0
10μM Rotenone:
10μM Quinone:
Id MnVK 1 2 4 5 6 8 14 15 16 17 18 19 20 21 22 23 24 25 26 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 53 54 55 56 57 58 59 60 61 62 64 65 66 67 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97
Treatment

(ii)

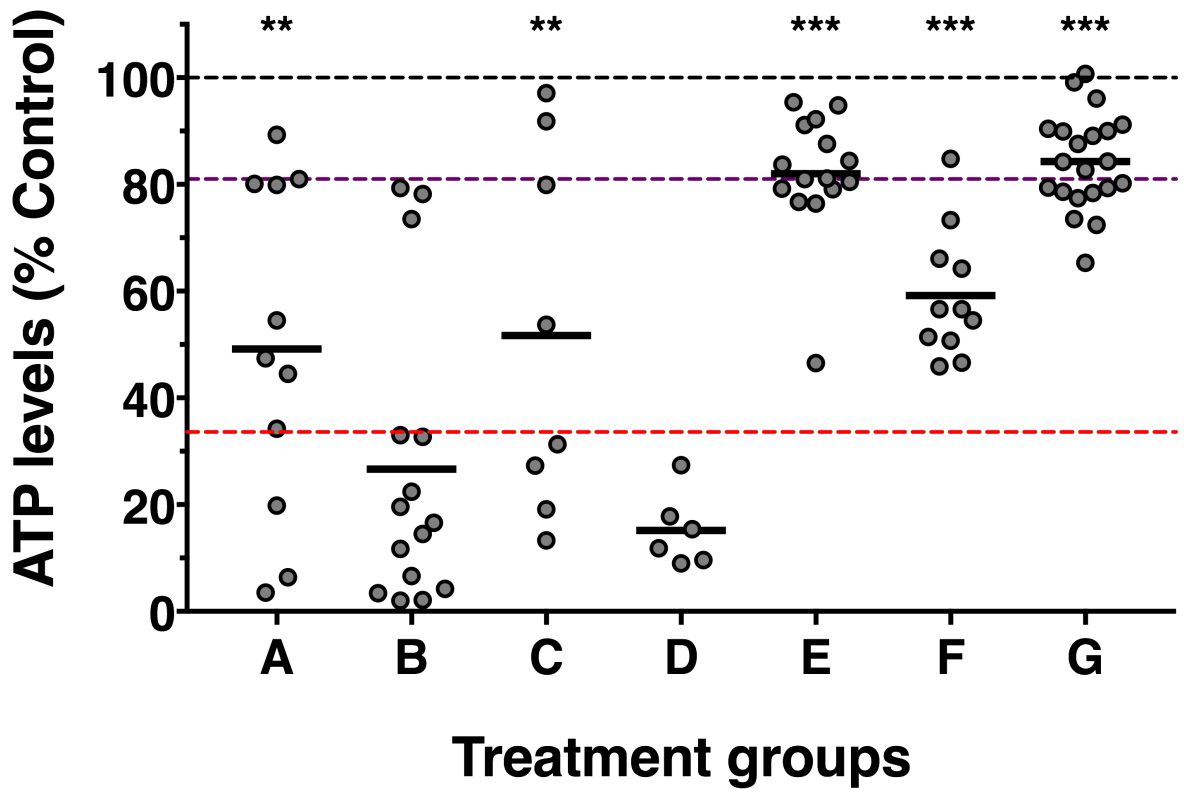


Figure 21: Acute rescue of adenosine triphosphate (ATP) levels by novel short-chain naphthoquinones (SCQs) in the presence of rotenone. Rescue of adenosine triphosphate (ATP) levels by test compounds (10 µM) in the presence of rotenone (10 µM) was measured and expressed as percentage of sham-treated (no rotenone or SCQ) HepG2 cells. Black, red and purple dotted lines represent the rescue of ATP levels in sham, rotenone and idebenone (Id, 10µM)-treated cells, respectively. **(i)** Pink data points represent the SCQs that improve viability significantly better than Id. Significance was calculated by using Student *t*-test (p^* <0.05, p^{**}<0.01, p^{***}<0.001 ATP rescue by SCQs against Id-treated cells, $p\#$<0.05 ATP rescue by SCQs against rotenone-only treated cells) **(ii)** Rescue of ATP levels by test compounds categorised according to their functional groups. Significance was calculated by using Student *t*-test. (p^* <0.05, p^{**}<0.01, p^{***}<0.001 ATP rescue by SCQs against rotenone-only treated cells).

Each data point represents one SCQ, data is expressed as mean ± S.D. of at least 3 independent experiments with 6 replicates within each experiment. Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

Mn, Menadione; VK, Vitamin K; A, Slight polarity; B, Aliphatic; C, Acid; D, Aliphatic ester; E, Amino ester; F, Amino acid; G, Amino alcohol.

2.4.1.4 Chemical reduction of SCQs to hydroquinone by NQO1 and other cellular reductases

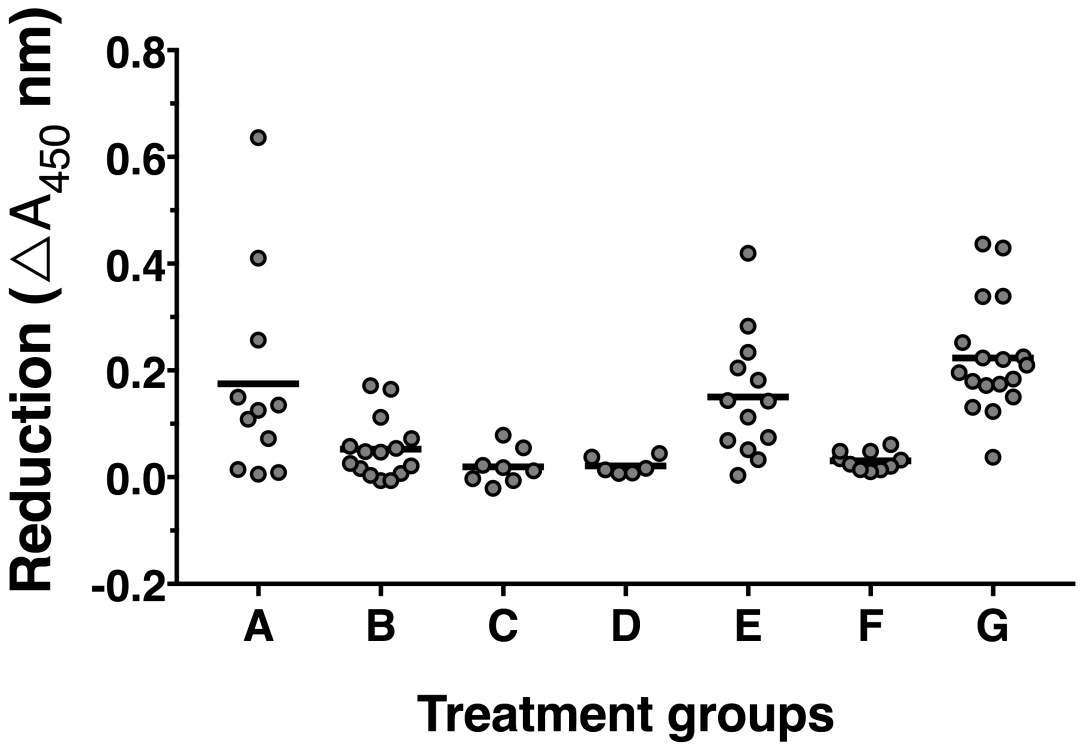
To measure *in vitro* reduction of novel SCQs by the cellular reductases, a colorimetric assay employing a water-soluble, cell-impermeable, electron accepting, tetrazolium dye (WST-1) was used. The reduction of quinones by cellular reductases was measured by a change in absorbance of the WST-1 dye (Figure 16). It was previously reported that the WST-1 dye can accept electrons generated from the reduction of quinones by cellular reductases to undergo an irreversible reduction to formazan. Therefore, the difference in absorbance of WST-1 corresponding to the reduction of dye directly reflects the reduction of quinones (Tan and Berridge 2004, Tan and Berridge 2010).

The reduction of SCQ by cellular reductases was calculated from the difference in absorbance of the WST-1 dye upon SCQ treatment ($\triangle A_{450}$ nm). When SCQs were categorised by their functional groups, the reduction of SCQs within A to G groups was 0.175 ± 0.19, 0.053 ± 0.057, 0.019 ± 0.033, 0.021 ± 0.016, 0.150 ± 0.115, 0.031 ± 0.017, 0.223 ± 0.104 respectively (Figure 22 i).

To understand if the reduction of SCQ was dependent on the reductase NQO1, NQO1 enzyme activity was inhibited by dicoumarol, as previously described (Haefeli et al. 2011). As previously reported, idebenone (Ide) is largely (93.9 %) reduced by the enzyme NQO1 (Figure 22 ii) (Haefeli et al. 2011). When the reduction of SCQs was compared, significant differences were observed with regards to their dependence on NQO1. Similar to idebenone, compounds #36, #40 and #41 were largely reduced by NQO1, while other compounds like #37 and #43 were equally reduced by other cellular reductases as well as NQO1 (Figure 22 ii). Interestingly, some compounds such as #1, #30, and #34 were

completely reduced in the absence of NQO1 activity (Figure 22 ii). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

(i)



(ii)

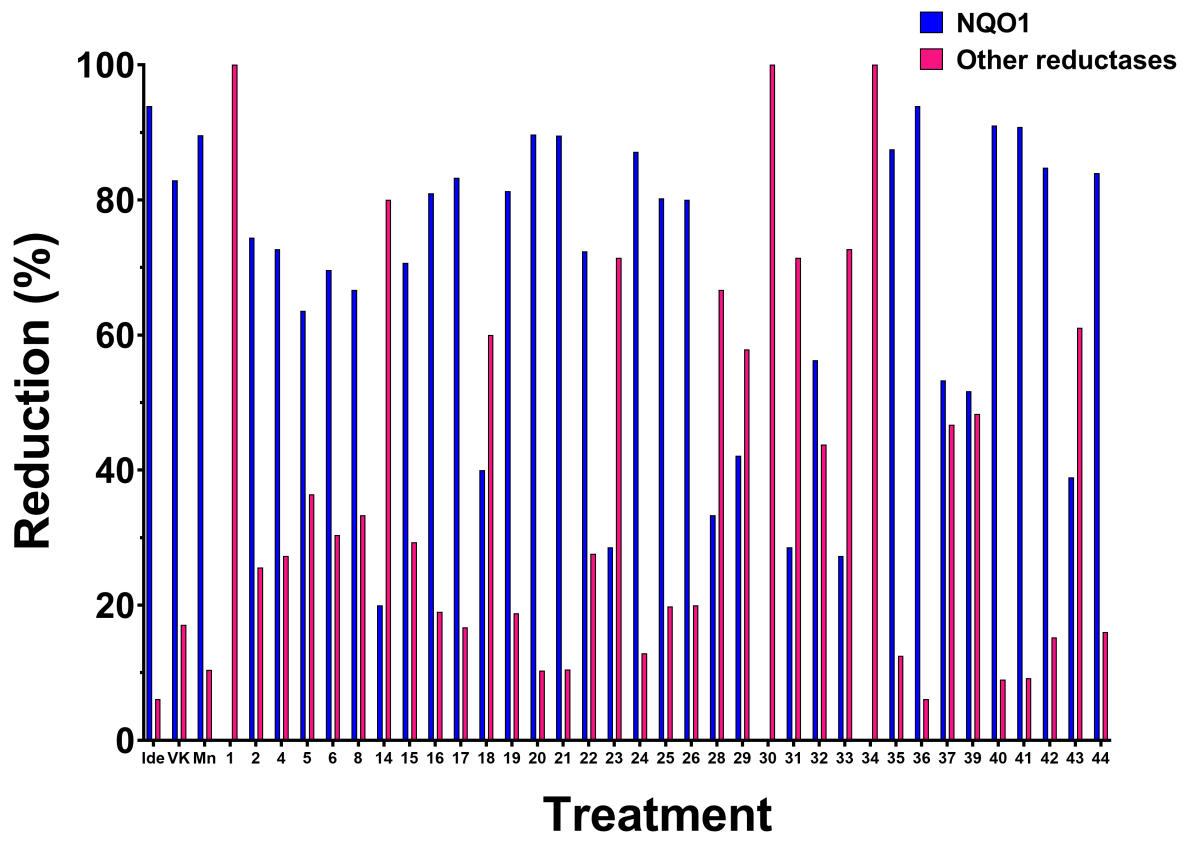


Figure 22: Bio-activation of novel short-chain naphthoquinones (SCQs) by cellular reductases. (i) Reduction of all SCQs by cellular reductases, categorised according to their functional groups. **(ii)** Reduction of few representative SCQs (10 µM) by NAD(P)H quinone oxidoreductase-1 (NQO1) was measured and expressed as percentage of total reduction. Each data point/bar represents the mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Complete data set for all test compounds are listed in appendix 2, Table A10-A16.

Ide, Idebenone; VK, Vitamin K; Mn, Menadione; A, Slight polarity; B, Aliphatic; C, Acid; D, Aliphatic ester; E, Amino ester; F, Amino acid; G, Amino alcohol.

2.4.1.5 The effect of novel SCQs on basal levels of cellular lipid peroxidation

Most of the SCQs that are developed as potential therapeutics are described to act as antioxidants and have been reported to prevent lipid peroxidation (James et al. 2005, Skulachev et al. 2009, Suno and Nagaoka 1984). However, there are some reports that SCQs can act as pro-oxidants at higher concentrations (Genova et al. 2003, King et al. 2009). To assess the pro/antioxidant capacity of our test compounds, HepG2 cells were treated with 10 μM test compounds for 72 h and basal lipid peroxidation (BLP) levels were measured using BODIPY-C_{11} dye. Although idebenone was previously reported to significantly reduce BLP levels down to 40 % in primary muscle cells (Erb et al. 2012), we only observed a reduction to 86 % in the HepG2 cells used in this study (data shown in appendix 2, Table A10). All novel SCQs were categorised by their functional groups to see the effects of tail chemistry on BLP. Although most SCQs appeared to slightly alter BLP levels, none of them showed any significant changes when compared to sham-treated cells (Figure 23). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

** One SCQ, #33, containing aliphatic ester attached to the side chain of the quinone core could not be tested for its effect on BLP levels due to unavailability of the compound for this assay.*

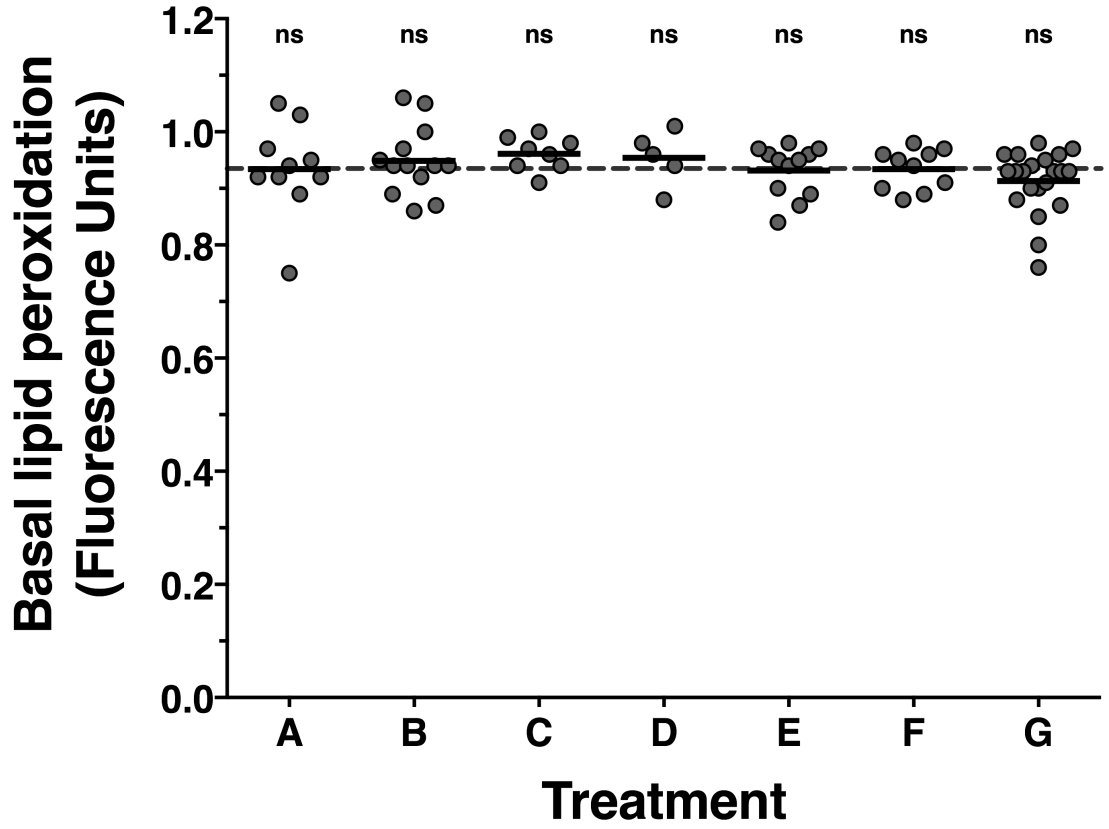


Figure 23: Effect of novel short-chain naphthoquinones (SCQs) on basal lipid peroxidation levels. Effect of test compounds (10 µM, categorised based on functional group, A-G) on basal lipid peroxidation levels. Statistical significance was calculated by using Student *t*-test to compare the effects of tail chemistry on lipid peroxidation against sham-treated cells (black dotted line). Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Data of individual test compounds are listed in appendix 2, Table A10-A16.

A, Slight polarity; B, Aliphatic; C, Acid; D, Aliphatic ester; E, Amino ester; F, Amino acid; G, Amino alcohol.

2.4.1.6 The influence of novel SCQs on extracellular lactate levels

Under normal conditions, cultured cells predominantly generate energy by glycolysis (Adam-Vizi and Chinopoulos 2006). This is not only a consequence of the fact that many cell lines are derived from cancer tissue, which inherently prefers glycolysis over OXPHOS. The reliance of adherent cultured cells on glycolysis is also a consequence of significantly reduced oxygen levels in cell culture where the cells consume oxygen faster that it can be supplied by diffusion alone (Kieninger et al. 2014). This effectively produces an aerobic zone surrounding the cells, unless the cell culture media is constantly moved (for example by excessive vibration within the incubator). To maintain glycolysis, cell require NAD^+ for converting glucose to pyruvate. Under conditions of mitochondrial dysfunction, the NAD^+ required to maintain glycolysis process is generated from NADH during the oxidation of pyruvate to lactate (Figure 8) (Heiden et al. 2009).

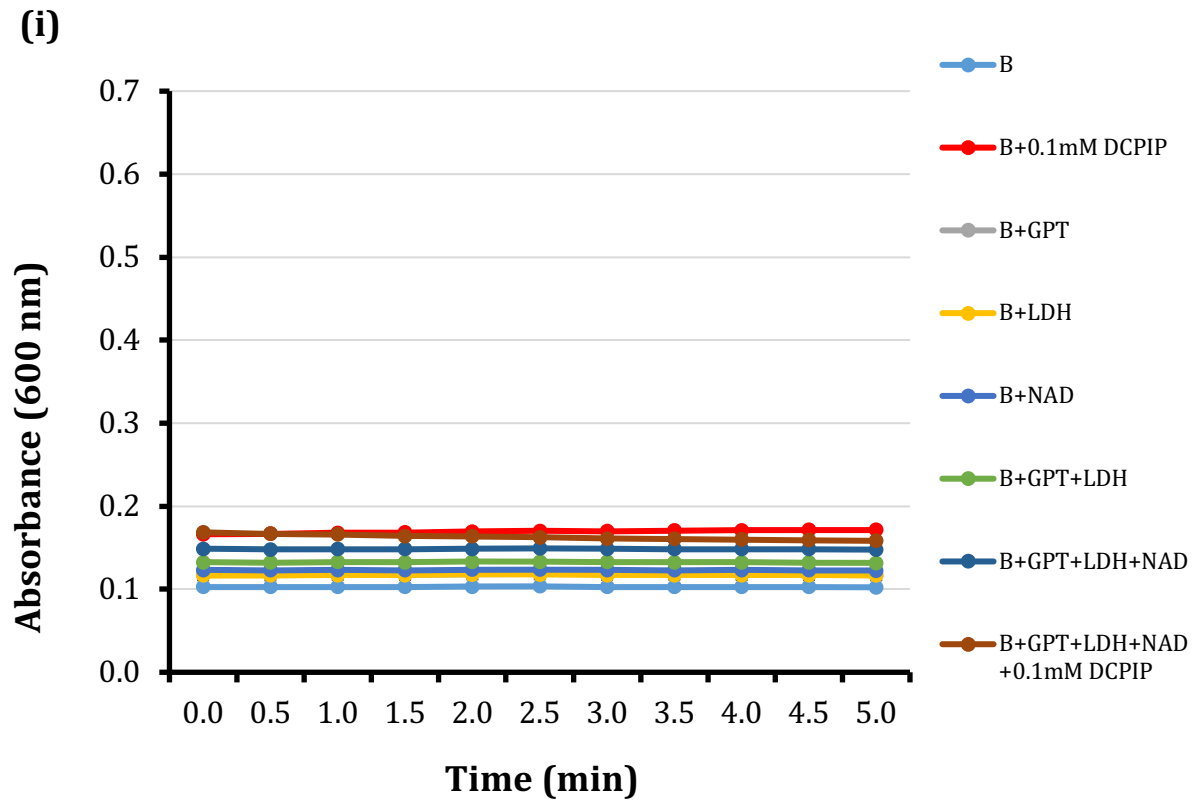
Quinones such as idebenone are reduced to the hydroquinone form in the presence of cellular reductases like NQO1, a process that also oxidises NADH to NAD^+ (Figure 17). This quinone-generated NAD^+ could theoretically be used to maintain glycolysis without the need to produce the potentially toxic lactate. Hence, it was of interest to understand if the novel SCQs could affect lactate levels (section 1.1.2.6). Alternatively, it was conceivable that the SCQs could increase glycolysis further to compensate for reduced mitochondrial ATP synthesis to protect cellular viability, which could result in increased lactate levels.

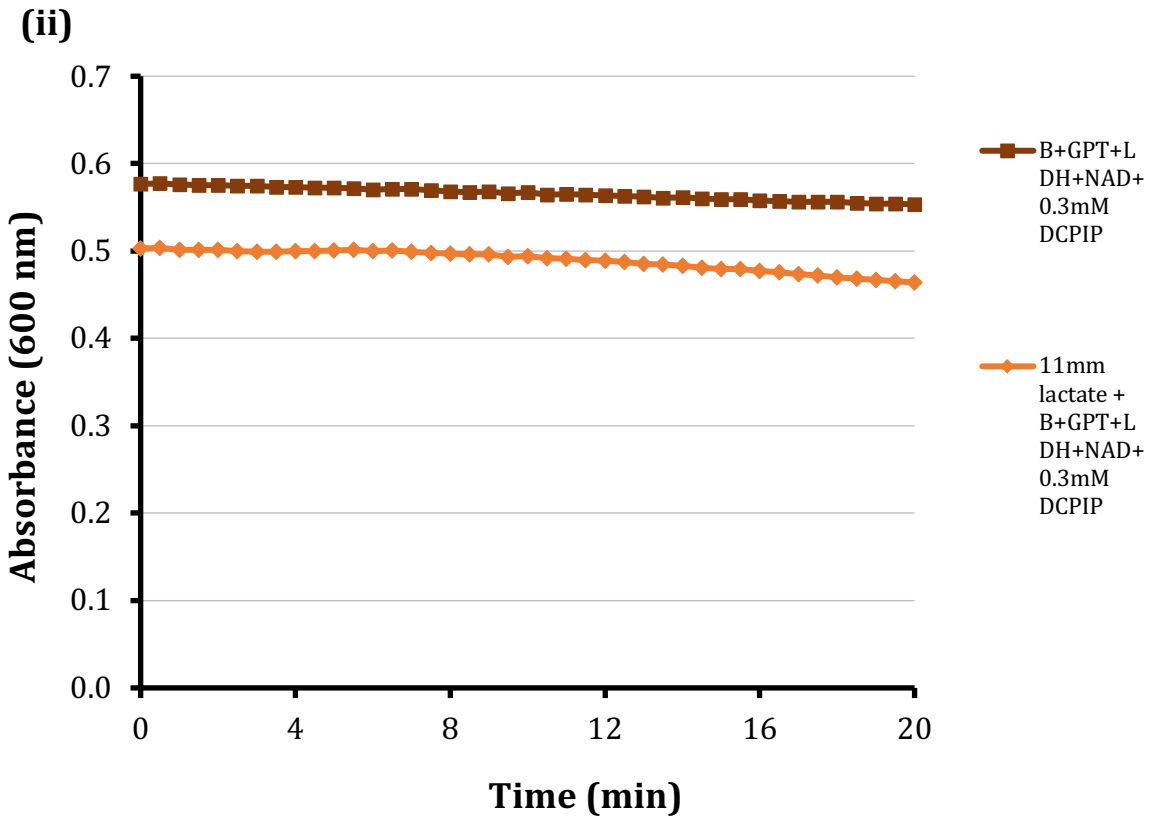
Developing an assay to measure cellular lactate levels

Lactate levels in the cell culture supernatant were measured by establishing a method reported previously (Dykens et al. 2008). To ensure no background activity was generated by any of the individual assay components, the change in absorbance of each component was initially measured separately at 600 nm. The base buffer (B, 100 µl, composed of 10 mM KH_2PO_4 buffer, pH 7.8 with 2 mM EDTA, 1 mg/ml BSA and 0.5 mM PMS) did not show any increase in absorbance (Figure 24 i). Stable absorbance was maintained with the individual addition of the dye DCPIP (0.6 mM), glutamate pyruvate transaminase (GPT, 5 U/ml), lactate dehydrogenase (LDH, 12.5 U/ml) and the electron acceptor NAD^+ (0.8 mM) to the base buffer. Subsequently, a combination of different components was tested including, base buffer with 5 U/ml GPT and 12.5 U/ml LDH; base buffer with 5 U/ml GPT, 12.5 U/ml LDH and 0.8 mM NAD^+; base buffer with 5 U/ml GPT, 12.5 U/ml LDH, 0.8 mM NAD^+ and 0.6 mM DCPIP. None of the combinations altered the absorbance of the reaction mixture at 600 nm (Figure 24 i) suggesting individual assay components did not change the absorption of the reaction mixture in the absence of lactate.

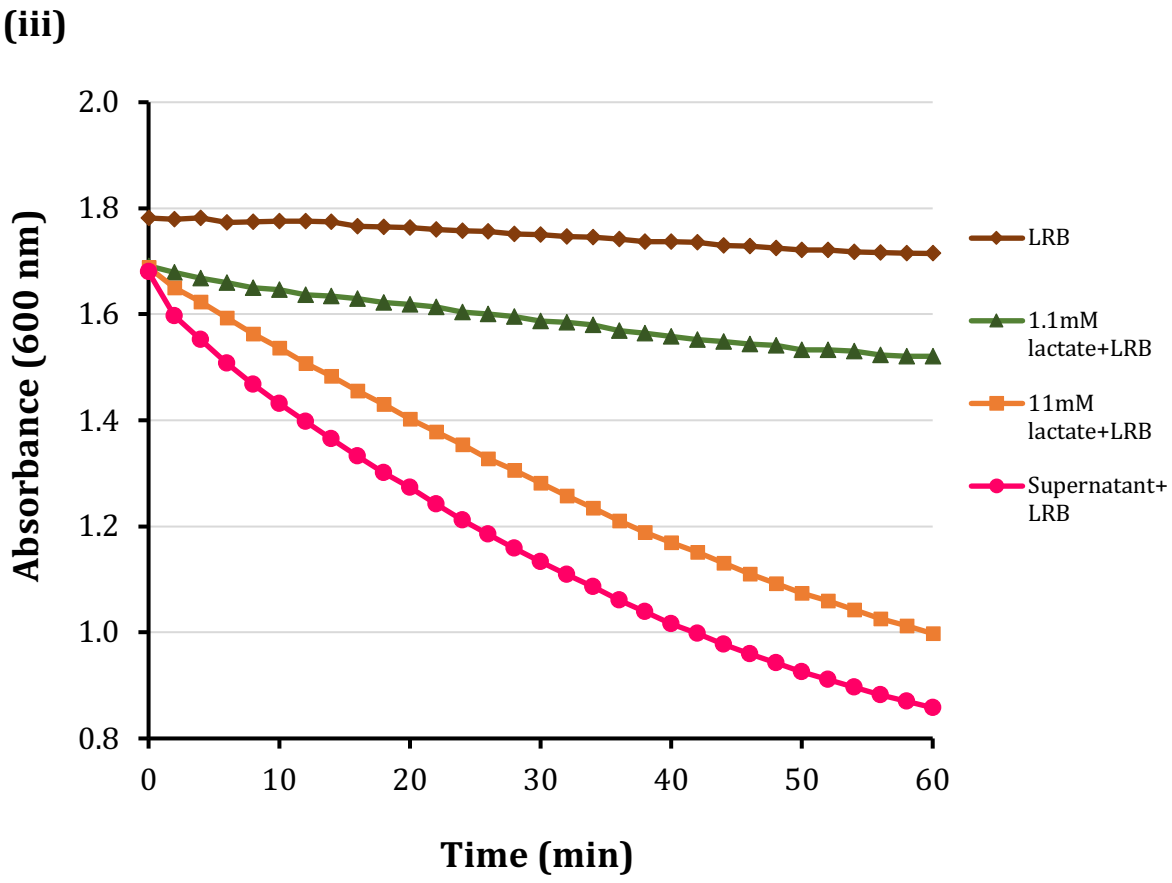
After confirming the lack of background activity in the base buffer, as a next step, the change in absorbance over time was measured, representing the rate of the reaction. The reaction was initiated by adding 10 µl of 11 mM lactate to 90 µl the reaction buffer containing 10 mM KH_2PO_4 pH 7.8, 1 mg/ml BSA, 0.5 mM PMS, 2 mM EDTA, 0.6 mM DCPIP, 0.8 mM NAD^+, 5 U/ml GPT, 1.5 mM glutamate and 12.5 U/ml LDH to measure. However, no change in absorbance was recorded (Figure 24 ii), which suggests that one or more components of the reaction buffer were inactivated, which prevented the reaction from proceeding. Since all enzymes and NAD^+ were added fresh to the reaction buffer before

every experiment, it was finally established that PMS was unstable over repeated freeze-thaw cycles. Therefore, all subsequent assays used freshly prepared PMS from a stock solution that was prepared in distilled in water rather than KH_2PO_4 buffer, as suggested by the manufacturer. The final lactate reaction buffer (LRB) consisted of 10 ml of 10 mM KH_2PO_4 pH 7.8, 1 mg/ml BSA, 0.5 mM PMS, 2 mM EDTA, 0.6 mM DCPIP, 0.8 mM NAD^+, 5 U/ml GPT, 1.5 mM glutamate and 12.5 U/ml LDH. After these changes to LRB, robust change in absorbance over time after the addition of 10 µl of 1.1 mM and 11 mM lactate to 90 µl of LRB were measured and a linear reaction rate was observed over 60 min (Figure 24 iii). Subsequently, supernatants from regular cell cultures were also measured (Figure 24 iii). A rapid decrease in absorbance, with a steeper slope compared to the 11 mM lactate standard was observed. Hence, the same method was followed for measuring lactate accumulation in cells after 72 h treatment with SCQs.




(ii)
Absorbance (600 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
4
8
12
16
20
Time (min)
B+GPT+L DH+NAD+ 0.3mM DCPIP
11mm lactate + B+GPT+L DH+NAD+ 0.3mM DCPIP

1


(iii)
Absorbance (600 nm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
10
20
30
40
50
60
Time (min)
LRB
1.1mM lactate+LRB
11mM lactate+LRB
Supernatant+ LRB

2

Figure 24: Method development for measuring cellular lactate in cell culture media. (i) The effect of individual components of the lactate reaction buffer on the final absorbance was measured by adding single component at a time to the base phosphate buffer (B). **(ii)** The effect of the addition of 11 mM lactate to the complete reaction buffer. **(iii)** The effect of the addition of lactate and supernatant from cultured cells to freshly prepared complete lactate reaction buffer (LRB). Each data point represents the average of three wells. Data represents the mean of three individual wells. Error bars omitted for clarity.

DCPIP, dichlorophenolindophenol; GPT, glutamate pyruvate transaminase; LDH, lactate dehydrogenase; NAD, nicotinamide adenine dinucleotide; DMEM, Dulbecco's modified eagle medium (complete media used for regular cell culture).

Influence of novel SCQs on basal cellular lactate levels

To get more insights into the influence of the novel SCQs on lactate levels, HepG2 cells were treated with novel SCQs for 72 h before the lactate accumulation was measured in the cell culture supernatant and normalised to protein content (Figure 25).

Using sham-treated cells, the lactate levels in cell culture supernatant were 67.3 ± 8.05 µmol/mg protein. When this was compared to the novel compounds, most SCQs altered cellular lactate levels with results ranging from 45 to 115 µmol/mg protein. However, some compounds from each groups A and B increased the lactate levels to more than 165 µmol/mg protein (Figure 25). On average, compounds from group D significantly increased lactate levels, whereas compounds from group F significantly decreased lactate levels, while the other categories of compounds did not induce any statistically significant changes (Figure 25). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

(Note: While I have established the complete assay to measure lactate levels, the influence of SCQs on lactate levels was conducted by a Master of Pharmaceutical Sciences student, Ayman LA Hemasa under my guidance. Data analysis was done by myself.)

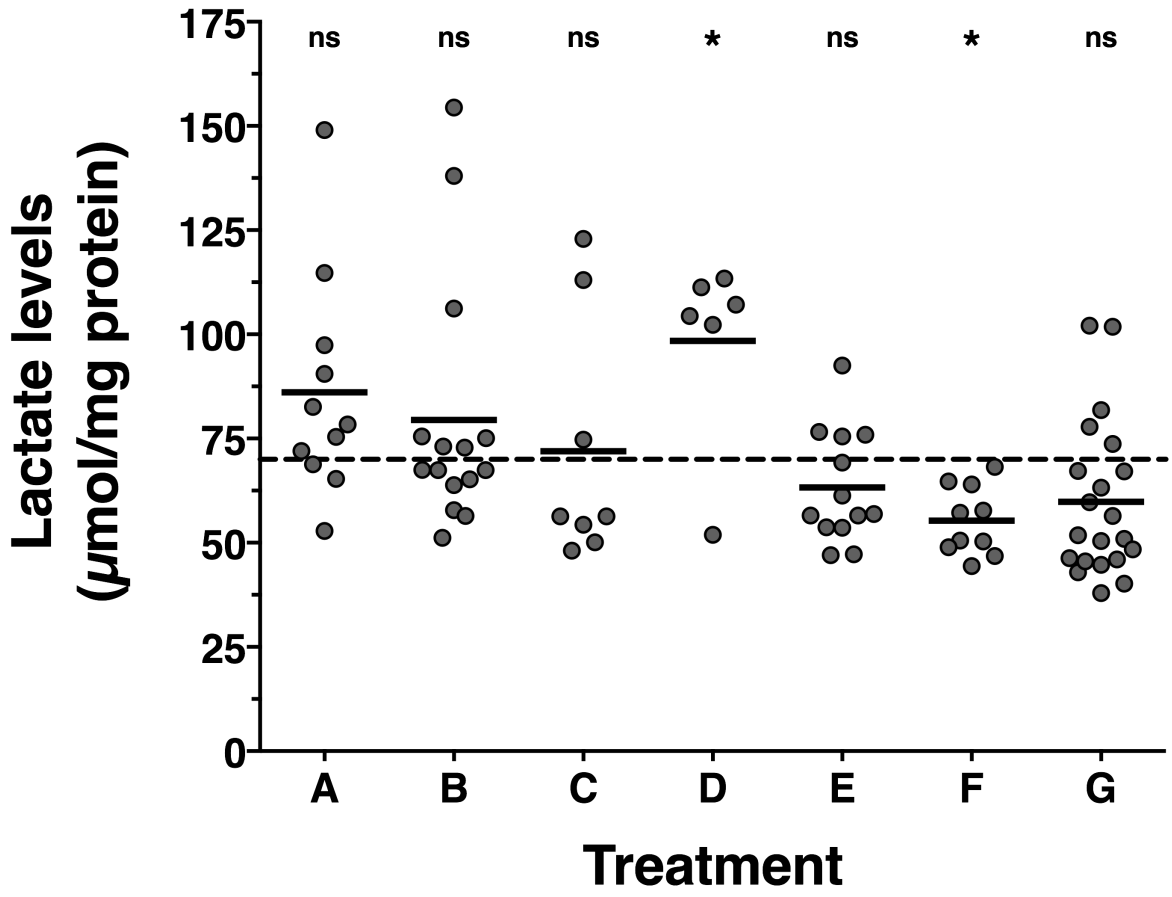


Figure 25: Effect of novel short-chain naphthoquinones (SCQs) on lactate levels in the cell culture media. Effect of test compounds (10 µM, categorised based on functional group, A-G) on cellular lactate levels, calculated as µmol/mg protein. Statistical significance was calculated by using Student *t*-test to compare the effect on lactate levels by the different groups of SCQs against sham-treated cells (black dotted line, $p^* < 0.05$). Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Data of individual test compounds are listed in appendix 2, Table A10-A16.

A, Slight polarity; B, Aliphatic; C, Acid; D, Aliphatic ester; E, Amino ester; F, Amino acid; G, Amino alcohol.

2.4.1.7 The effect of SCQs on β-hydroxy butyrate levels in cell culture supernatant

In the absence of carbohydrates, cells can also utilize fatty acids to generate ATP via the β-oxidation of lipids. Fatty acids are oxidised to acetyl-CoA by β-oxidation and the resultant acetyl-CoA can enter the Krebs cycle and react with oxaloacetate to form citrate. However, under conditions of increased production of acetyl-CoA in the liver that exceeds the amount of available oxaloacetate for entry into the Krebs cycle, ketogenesis is initiated resulting in the production of BHB, acetoacetate and acetone, collectively known as ketone bodies (Nelson et al. 2008, Gano et al. 2014). These ketone bodies are exported from the liver to tissues with high-energy demand such as the brain. The major ketone body BHB can be catabolized to acetyl CoA that can be further used for energy generation in tissues such as brain (Adeva-Andany et al. 2018). Apart from energy generation, BHB appears to have some protective activities. For example, a combination of BHB and acetoacetate (1 mM each) decreased oxidative damage and prevented neuronal death in rat neocortical neurons exposed to glutamate toxicity (10 μM) by increasing the oxidation of NADH in the mitochondrial ETC (Maalouf et al. 2007). Similarly, a high fat, low carbohydrate diet, known as ketogenic diet was shown to induce mitochondrial biogenesis, to decrease oxidative stress in mitochondrial myopathy and improve mitochondrial function (Ahola-Erkkilä et al. 2010, Milder and Patel 2012). Since ketone bodies are known to be predominantly metabolized in the mitochondria, the exact mechanism of how ketone bodies protect against mitochondrial dysfunction is unclear at present. One hypothesis simply involves increased NAD^+ levels as a consequence of BHB metabolism. In fact, this is a therapeutic strategy that is currently under clinical

investigation to counteract mitochondrial dysfunction and a range of other pathologies (see chapter 1.3.3).

It was therefore of interest to analyse if our novel SCQs increased concentrations of BHB in the cell culture supernatant to approach a mode of action for their cytoprotective activity (Figure 18).

Developing an assay to measure BHB levels in cell culture supernatant

To establish an assay to measure BHB in cell culture supernatant, I used a previous study as reference (Hansen and Freier 1978). The major components of the reaction mix contained PMS, NAD^+, BHB-dehydrogenase (BHBdh) and DCPIP as a colorimetric redox detector in Tris buffer, pH 7.8. The concentrations of individual components were selected based on my previous experience with the lactate assay (Section 2.4.1.6). Human blood normally contains <50 mg/ml BHB that can increase to >250 mg/ml under pathological conditions such as diabetic ketoacidosis (Elliott et al. 2010). This concentration range was used as a reference to determine the amount of BHBdh enzyme required, based on the enzyme units' information provided by the manufacturer (Sigma Aldrich, Castle Hill, Australia). The product information stated that one unit of BHBdh will oxidize 1.0 µmole of BHB to acetoacetate per min at pH 7.8 at 37 °C. Based on this information, the complete ketone reaction buffer (KRB) initially contained 0.5 mM PMS, 2.5 mM NAD^+, 0.0000625 U BHBdh, 0.2 mM DCPIP in 5 ml of 100 mM Tris HCl buffer (B), pH 7.8. As before, all components were separately tested for their influence on absorbance. Neither buffer alone (50 µl) nor buffer with the remaining KRB components

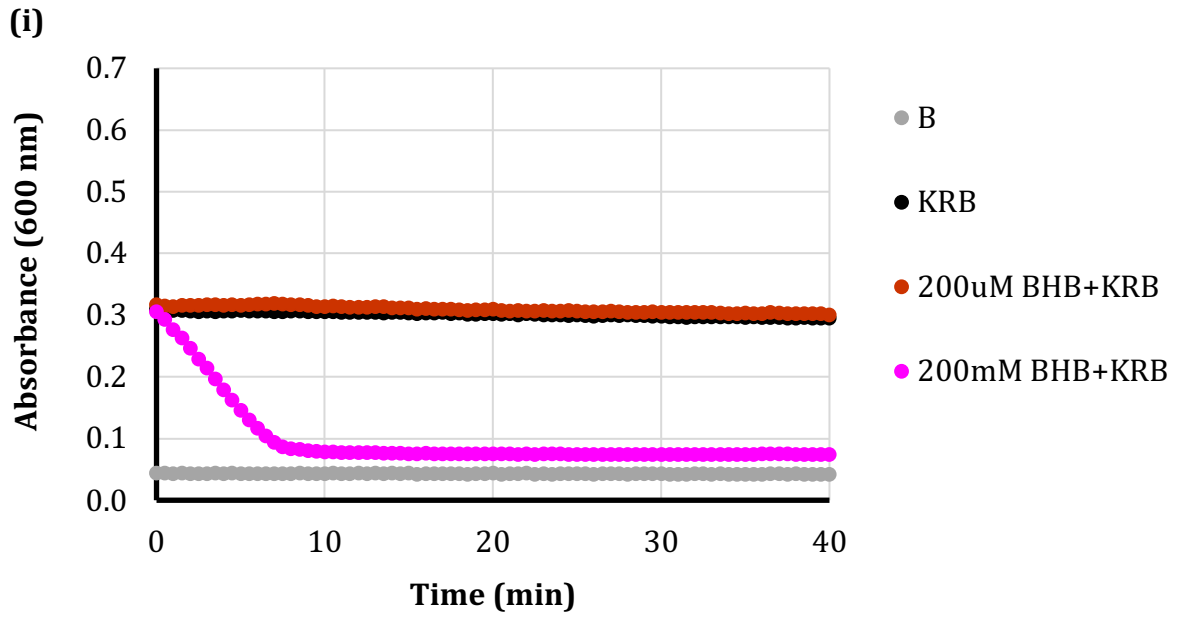
(50 µl total volume per well) changed the absorbance of the reaction mixture. Upon addition of 50 µl of 200 mM BHB standard per well to 50 µl of KRB, a clear decrease in absorbance was seen over a period of 40 min (Figure not shown).

After confirming a working reaction mixture, the concentrations of individual components were altered to see their influence on the rate of reaction. Firstly, the concentration of NAD^+ in the reaction mix was reduced to 1.25 mM and increased up to 10 mM while keeping the concentrations of other components constant. Increasing the concentration of NAD^+ above 2.5 mM did not alter the rate of reaction, hence this was chosen as the ideal concentration (Figure 26 ii).

Next, in the final reaction mixture (5 ml), the amount of enzyme BHBdh was increased up to 0.00625 U (in 50 µl KRB/well) keeping rest of the components constant. With increased BHBdh in the reaction mix, the rate of reaction increased upon addition of BHB. The highest concentration of enzyme (0.00625 U BHBdh in 50 µl KRB/well) was selected to improve the sensitivity of the assay to measure concentrations of BHB such as 0.3 mM (Figure 26 ii).

Finally, concentration of the electron transporter PMS was altered from 0.5 mM to 2.5 mM to determine its effect on the reaction rate. Unfortunately, increased PMS concentrations negatively affected the linearity of the reaction. Only the lowest concentration of PMS (0.5 mM) resulted in a linear reaction rate (over 10 min), and was therefore chosen for the final reaction mixture (Figure 26 ii).

Based on these results, the final KRB was composed of 5 ml of 100 mM Tris HCl buffer, pH 7.8 with 0.5 mM PMS, 2.5 mM NAD^+, 0.00625 U BHB-dehydrogenase and 0.2 mM DCPIP. This KRB (50 µl) was subsequently used to measure the effect of SCQs on the amount of BHB in 50 µl of cell culture supernatant.



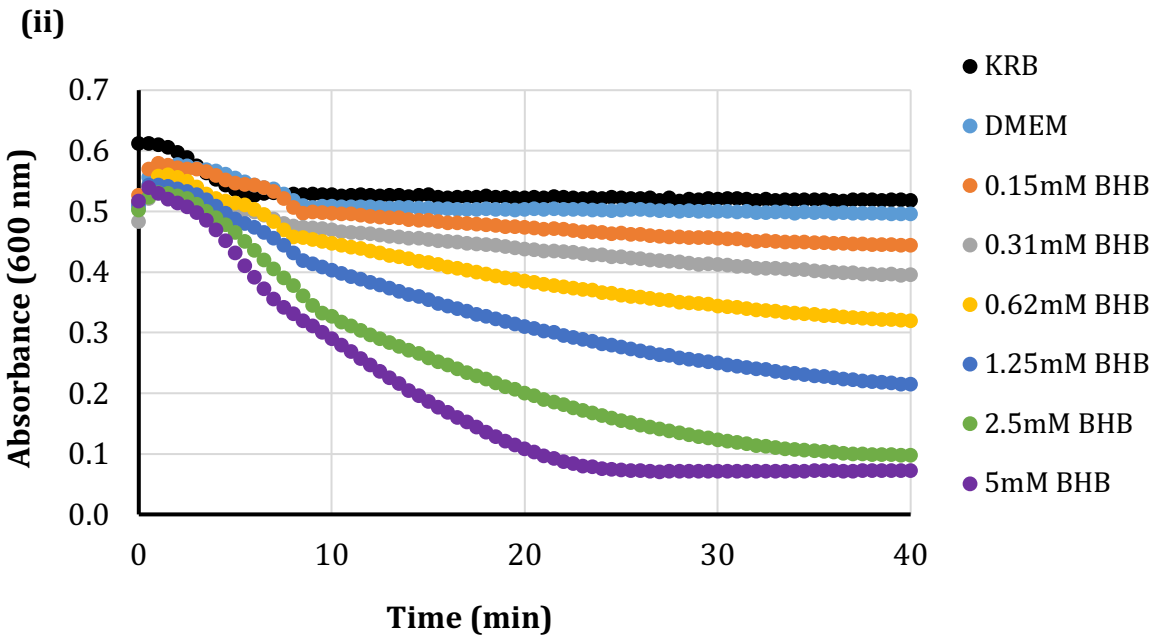


Figure 26: Method development for measuring β-hydroxybutyrate levels (BHB) in cell supernatant. (i) The effect of ketone reaction buffer (KRB) on the final absorbance. **(ii)** The effect of different concentrations of BHB on absorbance at 600 nm. Each data point represents the average of three wells. Error bars omitted for clarity.

B, Tris HCl buffer; DMEM, Dulbecco's modified eagle medium (complete media used for regular cell culture)

Influence of novel SCQs on BHB levels in cell culture supernatant

To measure the influence of the novel SCQs on BHB levels, HepG2 cells were treated with novel SCQs for 72 h before the BHB accumulation was measured in the cell culture supernatant and normalised to protein content (Figure 18).

Most of the compounds lead to approximately 1 to 3 µmol/mg protein in cell supernatant (Figure 27). However, one SCQ from each slightly polar (A), amino ester (E) group of compounds did increased BHB levels to more than 5 µmol/mg (5.37 ± 1.44, 5.14 ± 0.15 µmol/mg, respectively), when compared to sham-treated cells which averaged at 1.75 ± 0.41 µmol/mg protein. Overall, except for the aliphatic (B) and aliphatic esters (D), all other SCQs significantly increased BHB accumulation in cells (Figure 27). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

(Note: While I have developed the complete assay to measure ketone levels, the influence of SCQs on ketone levels was conducted by an Masters of Pharmaceutical Sciences student, Sueanne Chear, under my guidance. Complete data analysis was done by myself.)

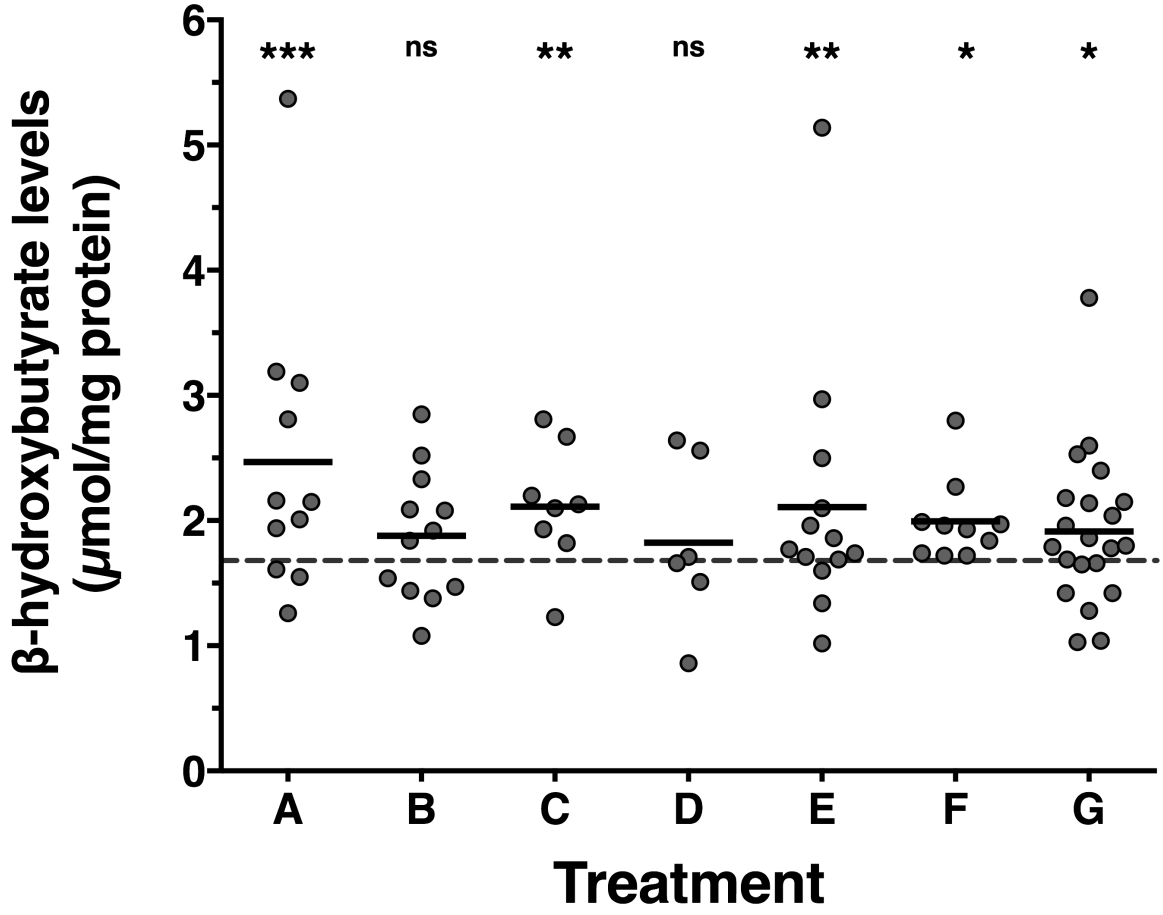


Figure 27: Effect of novel short-chain naphthoquinones (SCQs) on β-hydroxybutyrate (BHB) levels in cell culture supernatant. Effect of test compounds (10μM, categorised based on functional group, A-G) on BHB levels in cell supernatant, calculated as μmol/mg protein. Statistical significance was calculated by using Student *t*-test to compare the effect on BHB levels by SCQs against sham-treated cells (black dotted line, p^{*} <0.05, p^{**}<0.01, p^{***}<0.001). Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

A, Slight polarity; B, Aliphatic; C, Acid; D, Aliphatic ester; E, Amino ester; F, Amino acid; G, Amino alcohol.

2.4.1.8 Mitochondrial mass

During the growth and differentiation of normal cells, the total amount of mitochondrial organelles per cell (=mitochondrial mass) is continuously adapting to the cellular requirements (Heiden et al. 2009). In addition, under conditions of mitochondrial dysfunction, cells can upregulate mitochondrial mass (MM) as a compensatory mechanism to ensure metabolic and energy demands are met (Lee et al. 2002, Noh et al. 2013). This opens a therapeutic window as several drugs have been reported to influence MM. The most widely described classes of drugs with this activity are the fibrates and glitazones (Morel and Singer 2014). However, in primary human muscle cells, the benzoquinone idebenone was also able to increase mitochondrial mass in a dose-dependent manner (unpublished observation, N. Gueven).

To understand if the novel SCQs protect against mitochondrial dysfunction by affecting MM, HepG2 cells were treated with selected SCQs (10 μM) for 72 h before the MM was measured. Although the five selected SCQs along with idebenone (Ide), menadione (Mn) and vitamin K (VK) as control compounds appeared to slightly alter MM, none of these effects were statistically significant compared to sham-treated cells (Figure 28). Since the mitoprotective idebenone and randomly selected novel SCQs did not affect MM in these cells, this particular approach was suspended and the remaining SCQs were not tested.

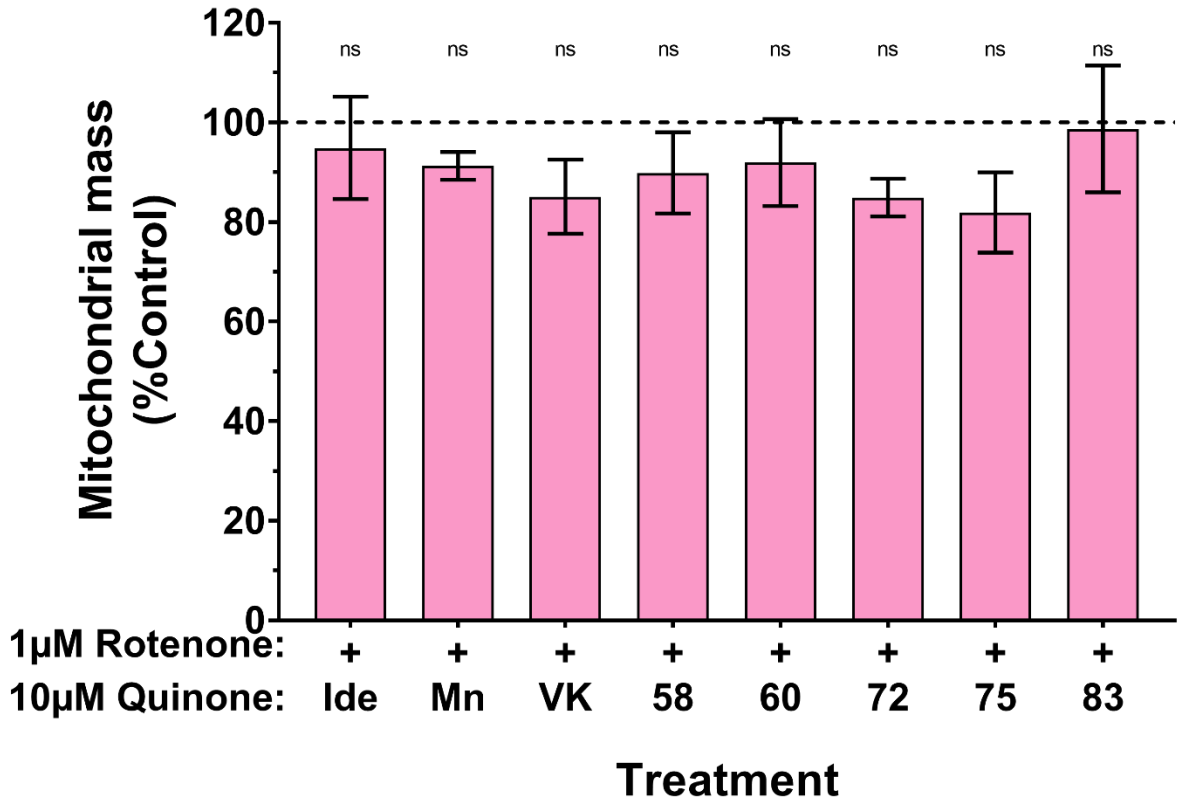


Figure 28: Effect of short-chain naphthoquinones (SCQs) on mitochondrial mass. Effect of selected SCQs (10 μM) on mitochondrial mass, calculated as mitochondrial mass/protein, expressed as percentage of sham-treated cells (Black dotted line). Statistical significance was calculated by using Student *t*-test to compare the effect on mitochondrial mass levels by SCQs against sham-treated cells ($p^* < 0.05$). Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments).

2.4.2 Functional activity correlations

2.4.2.1 Correlation of the cytoprotective ability and acute rescue of ATP levels by SCQs versus the compound's solubility

The solubility of compounds determines their ability to penetrate cells and hence affect their efficacy. The current library of novel SCQs was developed by modifying the functional groups attached to the side chain which was attached to the naphthoquinone core. Apart from changing the interaction of the molecule with any target proteins, these functional groups were also predicted to change the solubility of the compounds. For optimized cytoprotective activities, a previous report for short-chain benzoquinones reported a log D range between 2 and 4 (Erb et al. 2012). Therefore, it was important to analyse the influence of solubility of our naphthoquinones on their ability to protect against mitochondrial dysfunction.

When cytoprotection and acute rescue of ATP levels by SCQs in the presence of the mitochondrial inhibitor rotenone were correlated against compound solubility (log P and log D) no obvious correlations were observed (Figure 29). Surprisingly, in contrast to benzoquinones (Erb et al. 2011) a rather broad solubility range was associated with the protective activities of the naphthoquinone compounds of the current study. A compound solubility between log P = 1-7 and log D = 0-7 was associated with at least 60 % cytoprotection (Figure 29 i, iii) and 80 % of ATP level rescue (Figure 29 ii, iv). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

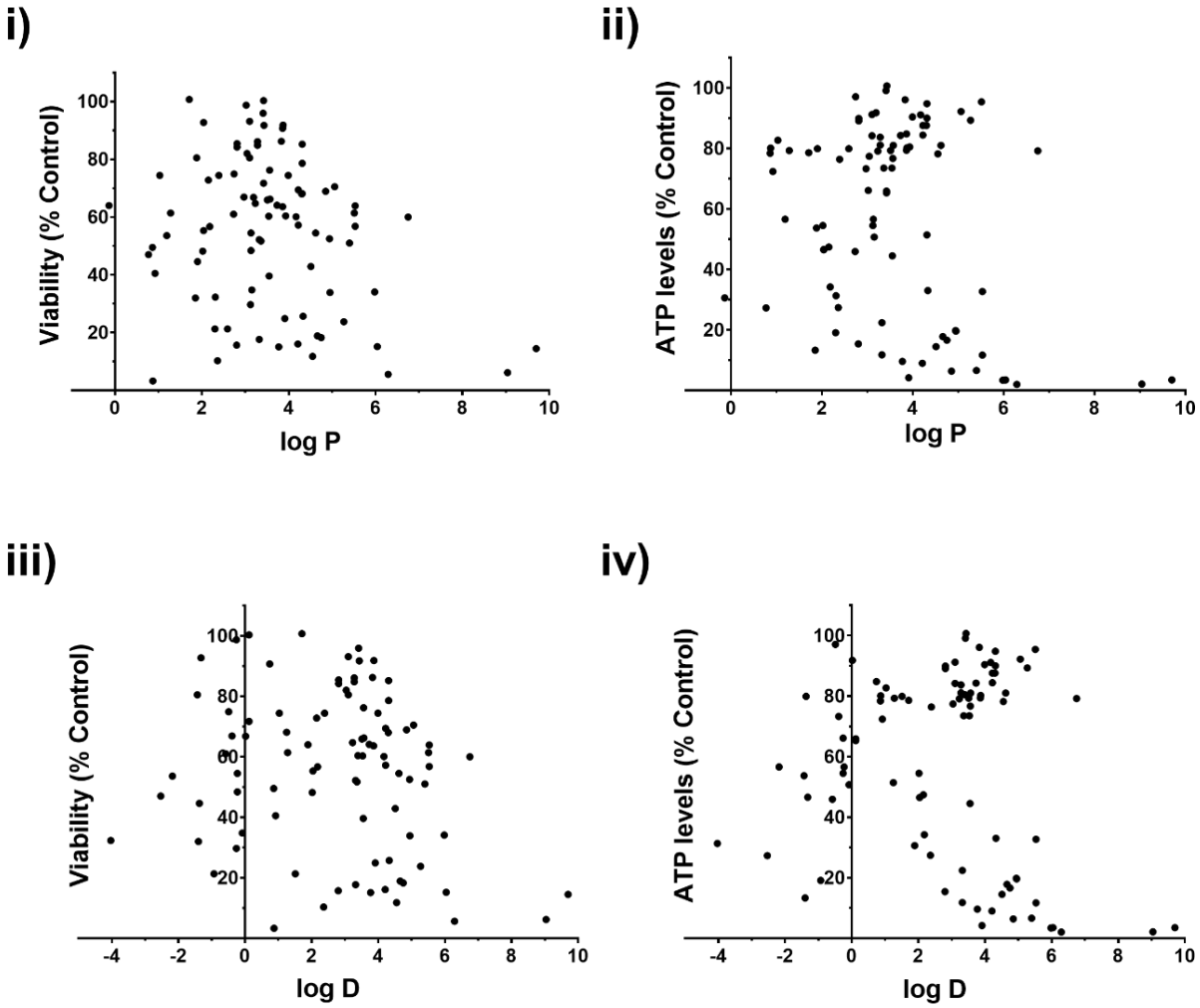


Figure 29: Correlation plot of cytoprotection and ATP rescue against solubility. (i-ii) Protection of cellular viability and acute rescue of ATP levels by test compounds (10 µM) in the presence of rotenone (1 µM and 10 µM respectively) correlated against logP. **(iii-iv)** Protection of cellular viability and acute rescue of ATP levels by test compounds (10 µM) in the presence of rotenone (1 µM and 10 µM respectively) correlated against logD. Each data point represents mean for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

2.4.2.2 Structure activity relationship of novel SCQs

The novel SCQs were generated in batches as part of a medicinal chemistry program where the results of the compound characterisation informed the chemical synthesis of the subsequent molecules. In particular the cytoprotection data for each compound directly led to the synthesis of new compounds with modified structures to improve their activity. Initially, the generated SCQs were grouped into slightly polar, aliphatic, acids and aliphatic esters based on the functional group attached to the side chain of the naphthoquinone core (Table 6) and tested for cytoprotection and ATP rescue. A broad grouping of the SCQs within each functional group was observed. While the slightly polar (brown squares, Figure 30) and acids (yellow squares, Figure 30) protected viability and rescued ATP to different degrees, the aliphatic compounds (green triangles, Figure 30) showed cytoprotection up to 60% of sham-treated cells but could not rescue ATP levels. On the other hand, the aliphatic esters (blue triangles, Figure 30) neither protected viability nor rescued ATP levels.

Based on these results, SCQs were optimized via an iterative process to achieve high levels of both cytoprotection as well as rescue of ATP levels. In order to increase the polarity of the compounds, amino acid fragments were added to the tail moiety and amino esters, acids and alcohols were generated. Clearly, the addition of the amino-functional groups increased the protective activity of the compounds. The most polar group, which contained amino acids (pink circles, Figure 30), protected viability between 30-100 %, but could not rescue ATP levels beyond 65 % compared to sham-treated cells. Slightly less polar amino esters (purple circles, Figure 30) and amino alcohols (red circles, Figure

30) showed consistently good levels of protection of both viability and ATP rescue. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

Therefore, more compounds with the amino acid and alcohols attached to the side chain were generated until consistently compounds were produced that protected cellular viability as well as rescued ATP levels to a high degree. This gave our team a clear understanding of the structure activity relationship of this class of compounds. It is important to note that the correlation plot (Figure 30) therefore illustrates the iterative optimization of compound activity, which led to the synthesis of more and more compounds that populated the top right-hand side corner of the plot. Therefore, despite of a subjective trend, the correlation plot of protection of viability versus acute rescue of ATP levels for all SCQs, does not demonstrate a correlation between the two parameters but is a consequence of repeated compound optimization (Figure 30).

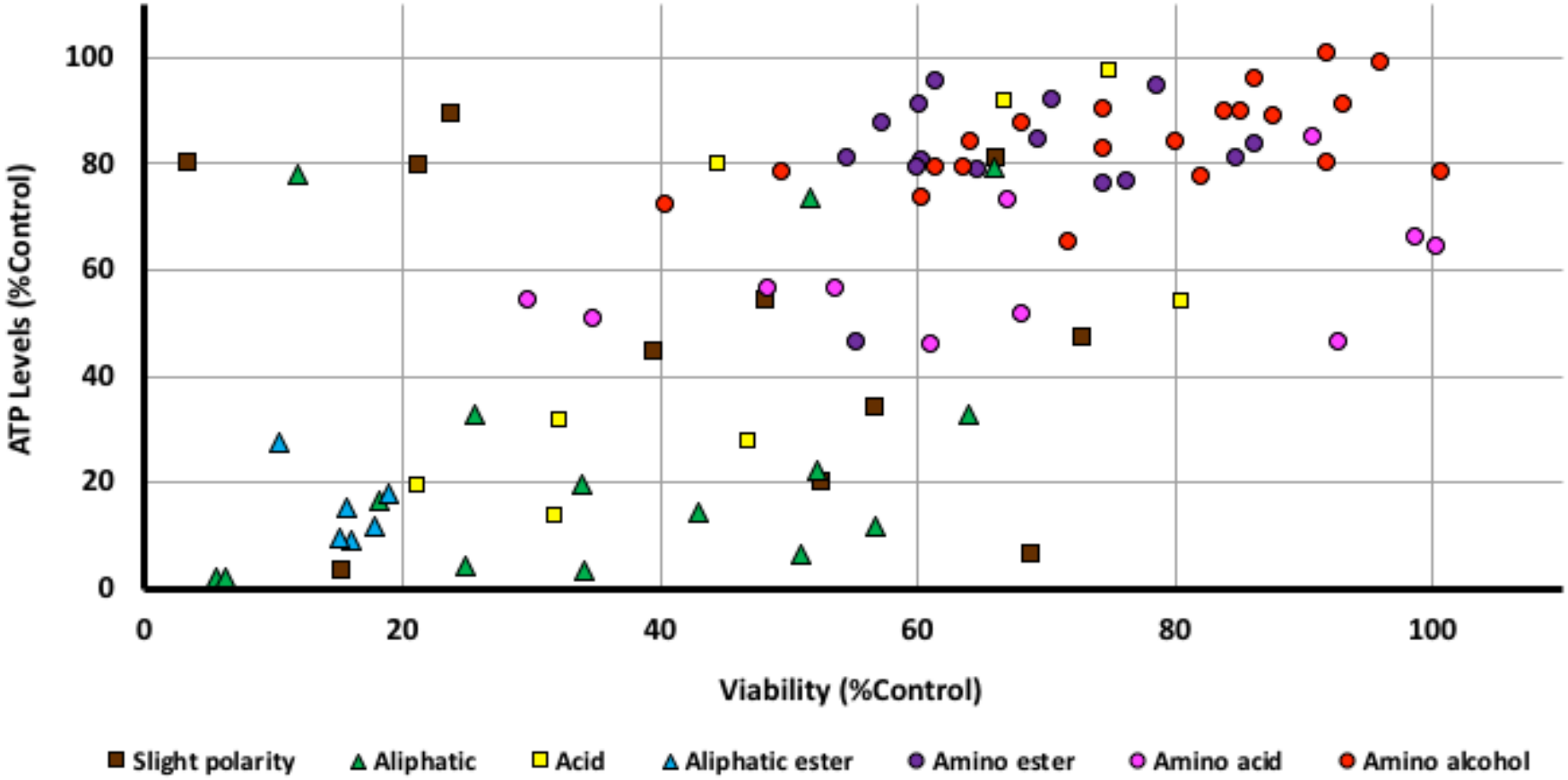


Figure 30: Correlation of cytoprotection by novel short-chain naphthoquinones (SCQs) versus acute rescue of adenosine triphosphate (ATP) levels in the presence of rotenone. Cytoprotection and acute rescue of ATP levels by test compounds (10 µM) in the presence of rotenone (1 µM, 10 µM respectively) was measured and expressed as percentage of sham-treated HepG2 cells. All SCQs are classified based on the functional group attached to the naphthoquinone core. Each data point represents one SCQ; data is expressed as mean ± S.D. of at least 3 independent experiments with 6 replicates within each experiment (n=3). Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16

2.4.2.3 Correlation of protection of viability versus the rate of reduction of SCQs by NQO1

Current opinion suggests that quinones should be bio-activated (reduced) from the quinone to the hydroquinone form to be able to protect against mitochondrial dysfunction (Haefeli et al. 2011, Prati et al. 2015). This would imply that compounds that are better reduced inside the cell should show more cytoprotective activity. Hence, to understand if the rate of reduction of novel SCQs by cellular reductases is proportional to the cytoprotective ability of the compounds, a correlation was plotted (Figure 31). However, in contrast to the hypothesis, enzyme-dependent reduction of quinones to their hydroquinone forms did not correlate with their cytoprotective activities (R^2 = 0.08, Figure 31). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

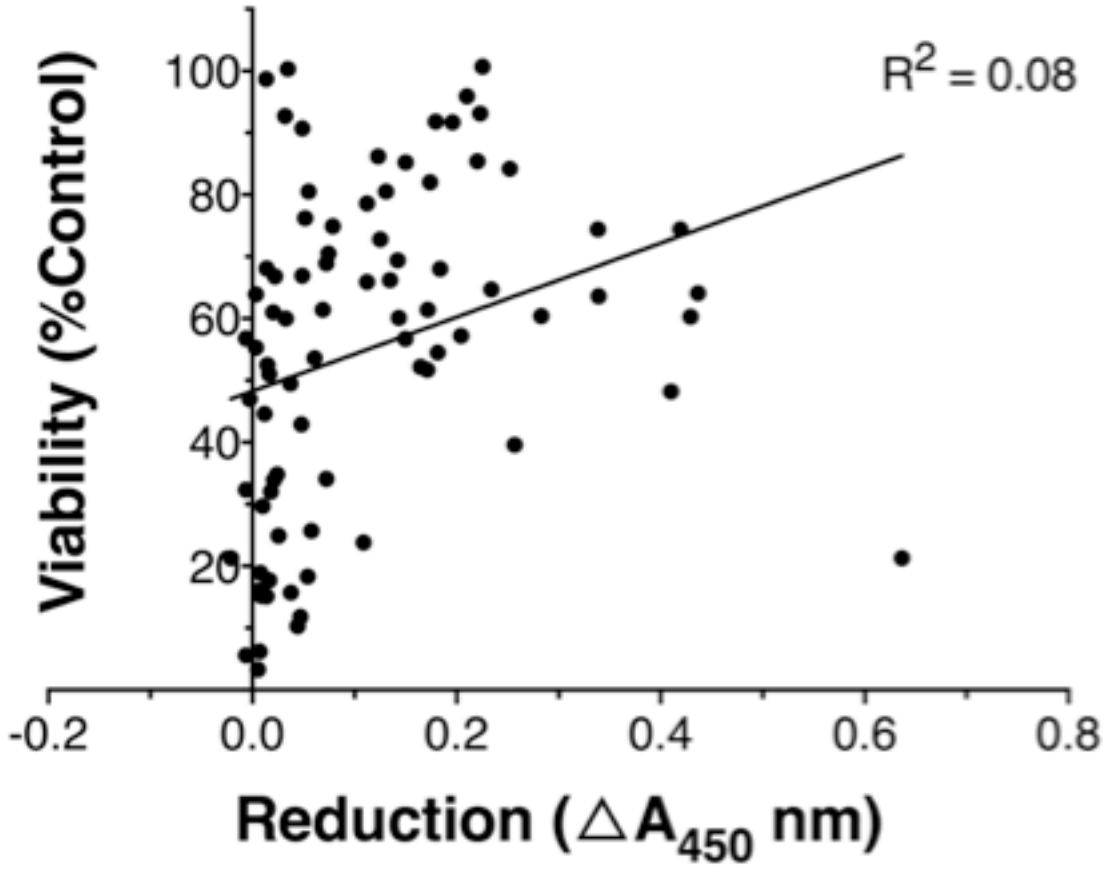


Figure 31: Correlation of cellular reduction of short-chain naphthoquinones (SCQs) by cellular reductases versus cytoprotection of cellular viability. Bio-activation of SCQs (10 µM) by cellular reductases was measured using a colorimetric assay (expressed as ∆ absorbance at 450 nm) and correlated against protection of cell viability by SCQs (10 µM) in the presence of rotenone (1 µM). Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

2.4.2.4 Correlation of antioxidant activity with cytoprotection

Most of the research on SCQs suggests that the antioxidant capacity of quinones is central for their cytoprotective activity against mitochondrial dysfunction (Ahmed 2014, Smith and Murphy 2010, Stelmashook et al. 2015). To understand if the antioxidant activity of SCQs to act as antioxidants was responsible for their cytoprotective capacity against

mitochondrial dysfunction, BLP levels were correlated with the cytoprotective activity of SCQs (both normalised to sham-treated cells) (Figure 32). This approach resulted in a random distribution of data points all across the graph, which indicated that these two parameters did not correlate (R^2 = 0.004, Figure 32). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

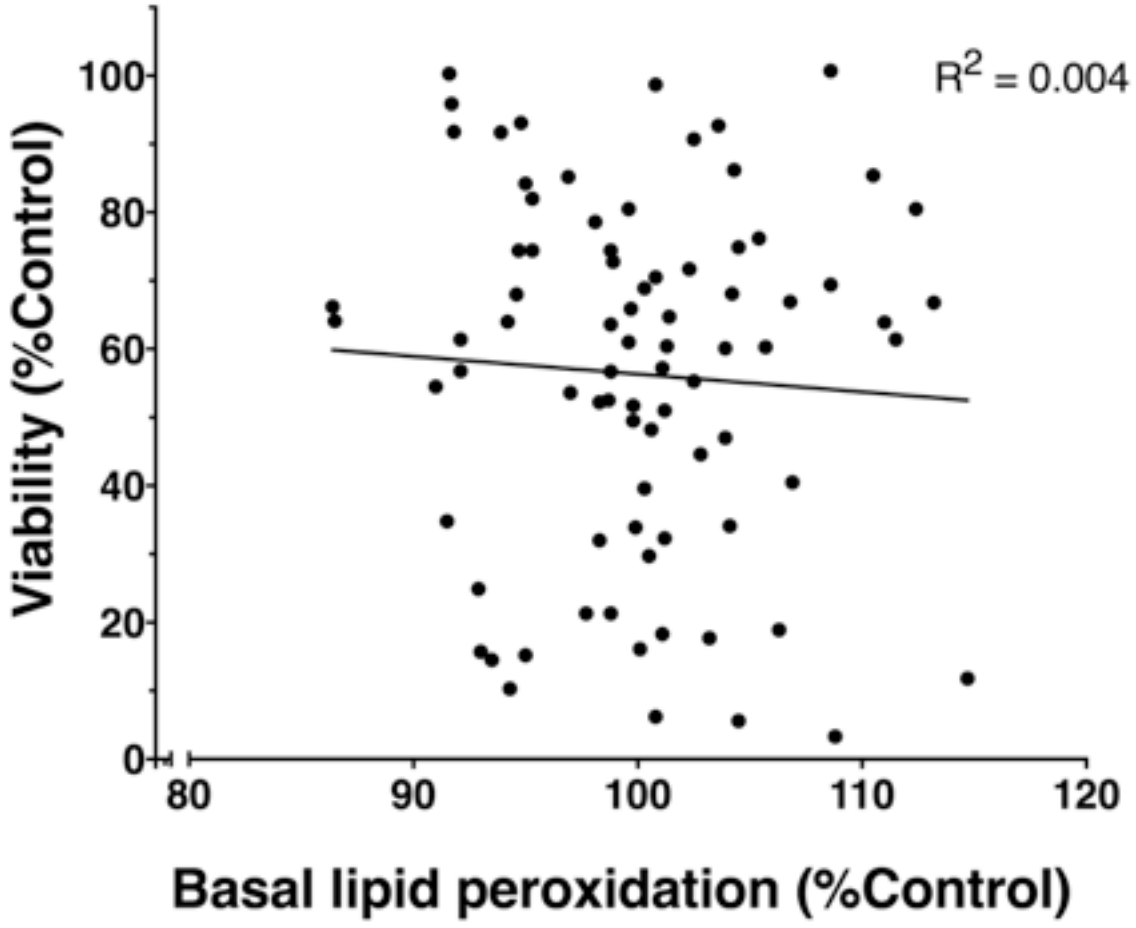


Figure 32: Correlation of cytoprotection and basal lipid peroxidation. The effect of short chain naphthoquinones (SCQs, 10 μM) on basal lipid peroxidation levels by NQO1 was measured (expressed as percentage of total reduction) and correlated against protection of cell viability by SCQs (10 μM) in the presence of rotenone (1 μM). Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

2.4.2.5 Correlation of cytoprotection with lactate or beta-hydroxy butyrate levels

To get insights into the mechanism of action of novel SCQs it was of interest to understand if the compounds affected glycolysis or beta-oxidation pathways of energy generation to protect against mitochondrial dysfunction. Hence, lactate and BHB levels were measured and correlated to cytoprotection for each compound (Figure 33).

Compounds that protected cellular viability to at least 60 % of control, which is similar to the cytoprotection shown by idebenone, affected lactate accumulation between 65 % to 140 %, compared to untreated cells. However, two compounds significantly increased lactate to 175 % of control cells (Figure 33 i). For compounds that protected viability to more than 70 % of control, which was significantly better than idebenone, the effect on cellular lactate levels ranged from approximately 65 % to 115 %, with one compound increasing lactate to about 140 %. As for previously tested parameters, no clear correlation between cytoprotection and lactate levels in the cell supernatant were observed (R^2 = 0.06, Figure 33 i).

A final option that was tested was the possibility that the novel compounds protected against mitochondrial dysfunction-induced cytotoxicity by upregulating ketone body synthesis. Most of the SCQs increased BHB accumulation in the cell culture supernatant (Figure 33 ii). However, these increases in BHB accumulation were clearly not associated with the cytoprotective effects of the SCQs (R^2 = 0.00003, Figure 33 ii). Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

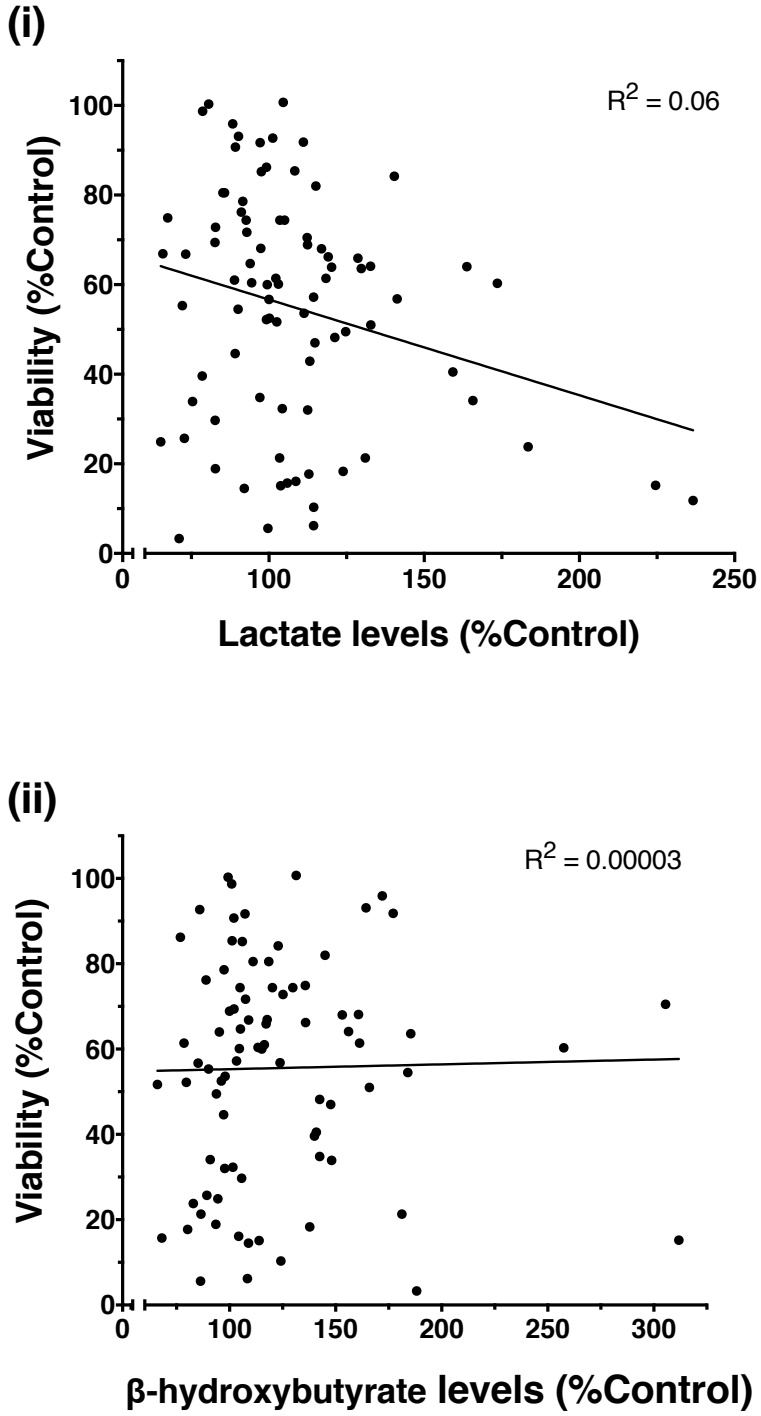
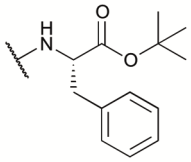
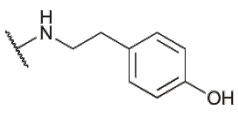
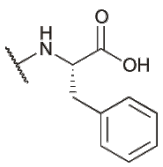
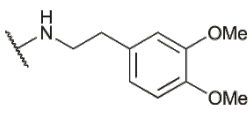
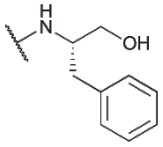


Figure 33: Correlation of protection of cellular viability by short-chain naphthoquinones (SCQs) versus their effect on cellular lactate and β-hydroxybutyrate (BHB) levels. Protection of cell viability by SCQs (10 μM) in the presence of rotenone (1 μM) is correlated to their effect on the accumulation of cellular lactate (i) and BHB (ii) levels. Each data point represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment). Error bars were omitted for clarity. Complete data of individual test compounds are listed in appendix 2, Table A10-A16.

2.4.3 Structural characterisation of novel SCQ's

Apart from characterising our novel SCQs for their ability to influence cellular parameters, structural aspects were also studied to understand the importance of the design of the quinone core structure. For this characterisation, five SCQs, with different functional groups attached to the side chain (R2-R6, Table 7), which protected viability significantly better than idebenone were chosen. In addition, one basic SCQ, where the quinone core was attached with a four-carbon (4C) side chain ending in hydroxyl group, without any functional group at the end was also included (R1, Table 7). Individual components of the structures of these selected SCQs were modified to analyse their effect on the compound's ability to protect cellular viability (Complete structures listed in Appendix 1, Table A8-A9).

Table 7: Structures of the side-chains of selected novel SCQs

R1		**R4**	
R2		**R5**	
R3		**R6**	

2.4.3.1 The relevance of the naphthoquinone core for cytoprotection

The major problem with currently researched and marketed ubiquinones like CoQ_{10} and idebenone is their solubility and their requirement to be bio-activated by cellular reductases like NQO1. Plastoquinones like SkQ1 and vitamin E derivatives such as EPI-743 reportedly have to be bio-activated by NQO1 as well to exert their protective potential (Anisimov et al. 2008, Enns et al. 2012, Manskikh et al. 2015, Skulachev et al. 2009). However, inactivating NQO1 polymorphism are present to a significant degree in the general population (Lajin and Alachkar 2013). In these individuals it would therefore be expected that these compounds cannot be bio-activated and instead undergo a one-electron reduction to the unstable semiquinone that gives rise to oxidative damage (Martin et al. 2009).

To address this issue, in this study, novel SCQs were developed with the aim to increase solubility and bioactivation. In particular, we aimed to generate compounds that could be reduced by other cellular reductases apart from NQO1, to differentiate these molecules from existing ubiquinones and plastoquinones. To address this aim, it was important to understand whether the presence of the functional group of the side chain or the naphthoquinone core or both of them were responsible for the cytoprotective ability of the novel SCQs. Therefore, five of the most cytoprotective SCQs were chosen and their quinone core was modified from a naphthoquinone to a plastoquinone and ubiquinone core while keeping the side chain (4-carbon, 4C) and the functional groups (R1-R6) identical (Figure 34, Table 7). Complete list of structures located in Appendix 1, Table A8.

For the set of compounds with R1, the quinone core is attached with a 4C side chain ending in hydroxyl group, without any functional group at the end. In this group, none of

the three compounds protected cellular viability against rotenone (Figure 34, red dotted line). This suggested that the quinone core with the 4C side chain did not have any cytoprotective ability in the absence of a functional group at the end (Figure 34). Interestingly, in four out of the five groups of compounds with the R2, R3, R5 and R6 functional groups, the naphthoquinone core showed significantly better cytoprotection compared to the corresponding plastoquinone and ubiquinone derivatives (Figure 34). In fact, in these four groups (R2, R3, R5 and R6), changing the core from naphthoquinone moiety to any of the other two quinone moieties significantly increased the cytotoxicity of these compounds. These changes produced compounds that significantly reduced viability below levels of rotenone-only treated cells in R2, R3 and plastoquinone from R6 group (Figure 34). In the group of compounds with the R4 functional group, the compound with the plastoquinone core protected cell viability significantly better compared to the corresponding naphthoquinone (Figure 34).

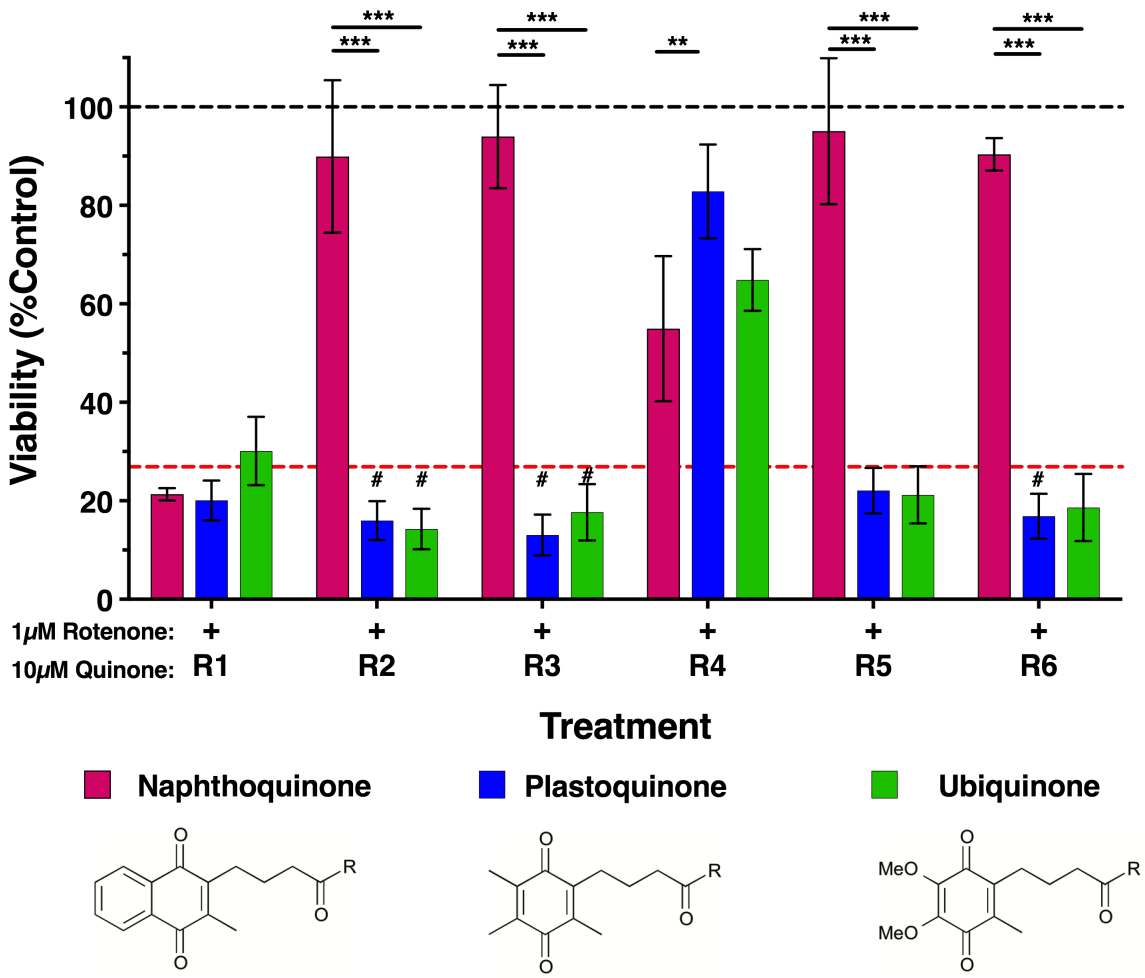


Figure 34: Cytoprotection by related quinones with different quinone cores in the presence of rotenone. Side chain (R, R1-R6, Table 6) from select naphthoquinones (pink bar) was attached to different cores, i.e. plastoquinone (blue bar) and ubiquinone (green bar). Cytoprotection by these test compounds (10 µM) in the presence of rotenone (1 µM) was measured and expressed as percentage of sham-treated (black dotted line) HepG2 cells. Each bar represents the activity of one compound, data is expressed as mean ± S.D. (n=3 independent experiments with 6 replicates within each experiment). Significance was calculated by using Student *t*-test (p^* <0.05, p^{**}<0.01, p^{***}<0.001 cytoprotection by naphthoquinones vs the related plastoquinones and ubiquinones, $p\#$<0.05 cytoprotection by SCQs against rotenone-only treated cells (red dotted line)).

2.4.3.2 The relevance of the methyl group on the naphthoquinone core

Most of the currently researched quinones including CoQ10, idebenone, EPI-743 and MitoQ have a methyl group attached to the quinone core at position X (Figures 13, 35), in contrast to SkQ1 that lacks this methyl group (Figure 13). To understand the importance of a methyl group (Me) at that particular position, it was replaced with either by hydrogen (H) or a hydroxyl (OH) group on the selected SCQs containing the R1-R5 functional groups attached to their side chain (see appendix 1, Table A9 for structures). In four of the five groups of naphthoquinones (R2-R6), the compounds with the Me group in the X-position showed significantly better cytoprotection when compared to their corresponding compounds with H and OH groups (Figure 35). When the Me group was replaced with the OH group in the compounds with the R5 functional group, a significant increase in cytotoxicity was observed for these compound (10.4 ± 4.6 %), compared to rotenone-only treated cells (Figure 35). Unfortunately, in the group of compounds with the R6 functional group, the naphthoquinone with the OH group in the X-position could not be synthesised and was therefore not tested (Figure 35). Surprisingly, only for compounds with the R1 group, the replacement of the Me group with H significantly improved cell viability (Figure 35). However, with 44.55 ± 15.04 % viability, this compound still showed less protection than idebenone (66.2 ± 12.05 %). In contrast, replacing the Me group with a OH group in the compound with the R1 group, reduced the cell viability to 15.6 ± 6.8 %, which was significantly lower than rotenone-only treated cells (red-dotted line, Figure 35). Consistently, for all synthesized compounds, the addition of the OH-group in the X-position increased cytotoxicity.

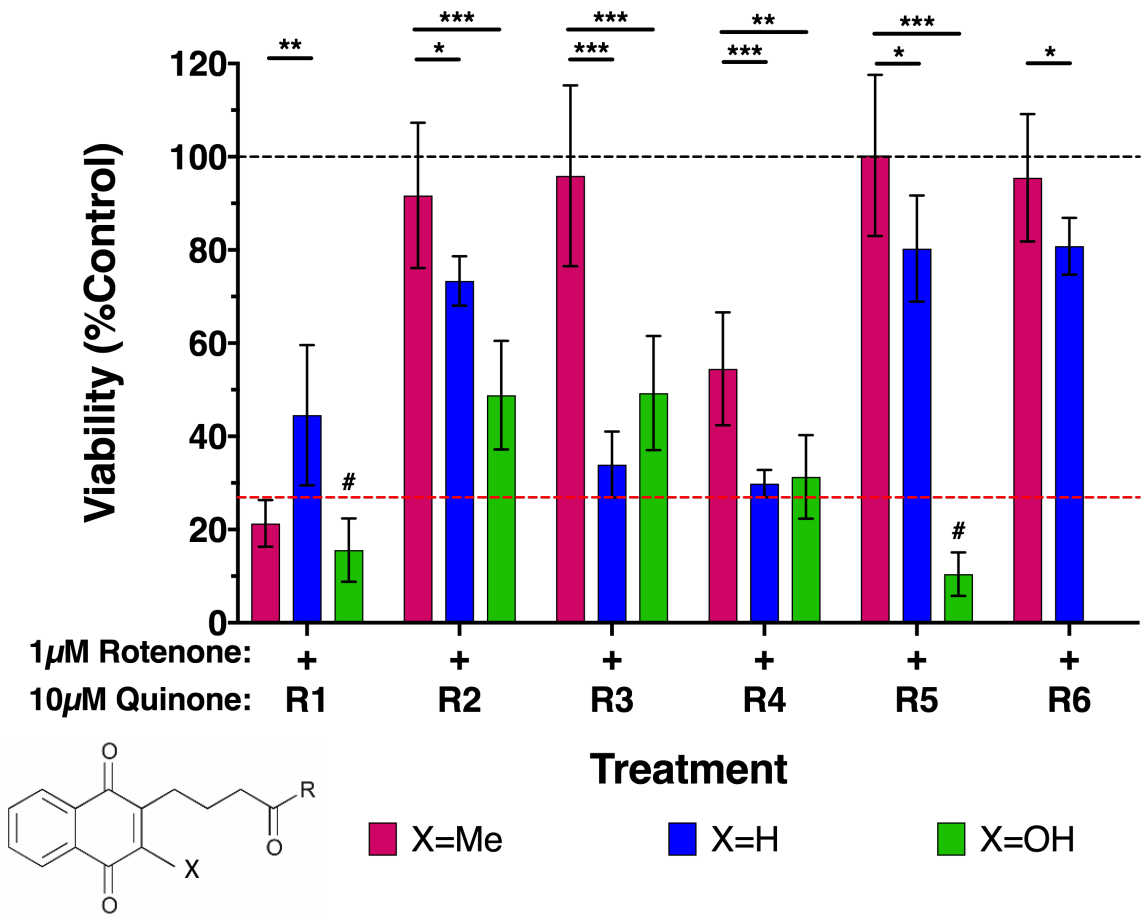
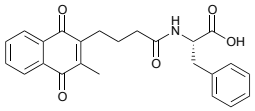
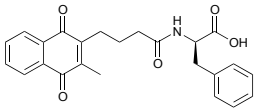
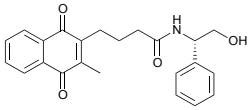
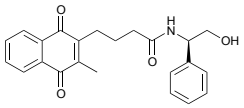
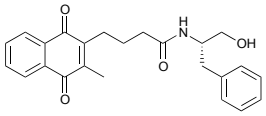
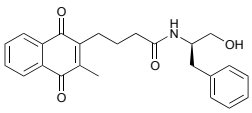
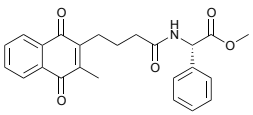
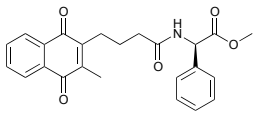
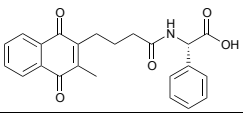
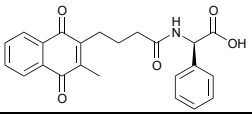


Figure 35: Effect of the functional group in the X-position for cytoprotection. The functional group located in the X-position of selected SCQs (R1-R6, table 6) was replaced from the original methyl group (Me, pink bar) to hydrogen (H, blue bar) or a hydroxyl group (OH, green bar). Cytoprotection by these test compounds (10 µM) in the presence of rotenone (1 µM) was measured and expressed as percentage of sham-treated HepG2 cells (black dotted line). Each bar represents the activity of one compound, data is expressed as mean ± S.D. (n=3 independent experiments with 6 replicates within each experiment). Significance was calculated by using Student *t*-test (p^* <0.05, p^{**}<0.01, p^{***}<0.001 cytoprotection by naphthoquinones containing Me group vs the related naphthoquinone with H and OH group at the X-position, $p\#$<0.05 cytoprotection by SCQs against rotenone-only treated cells (red dotted line)).

2.4.3.3 Effect of isomerization of the side-chain residue on cytoprotection

To evaluate the importance of spatial orientation of the functional group attached to the naphthoquinone core, five naphthoquinones were selected and their stereoisomers for the side-chain group were synthesised (Table 8). In four out of the five tested pairs of compounds, no significant cytoprotective differences were observed between the two stereoisomers. However, in one pair, the L-isomer (#37) showed significantly better cytoprotection (100.3 ± 17.3 %) when compared to its corresponding D-isomer (#94, 58.7 ± 5.2 %) (Figure 36).

Table 8: Structures of the corresponding pairs of short-chain quinones (SCQ) that were synthesised as L- and D-isomers

SCQ	L-isomer	SCQ	D-isomer
#37		#94	
#81		#80	
#62		#78	
#95		#97	
#96		#98	

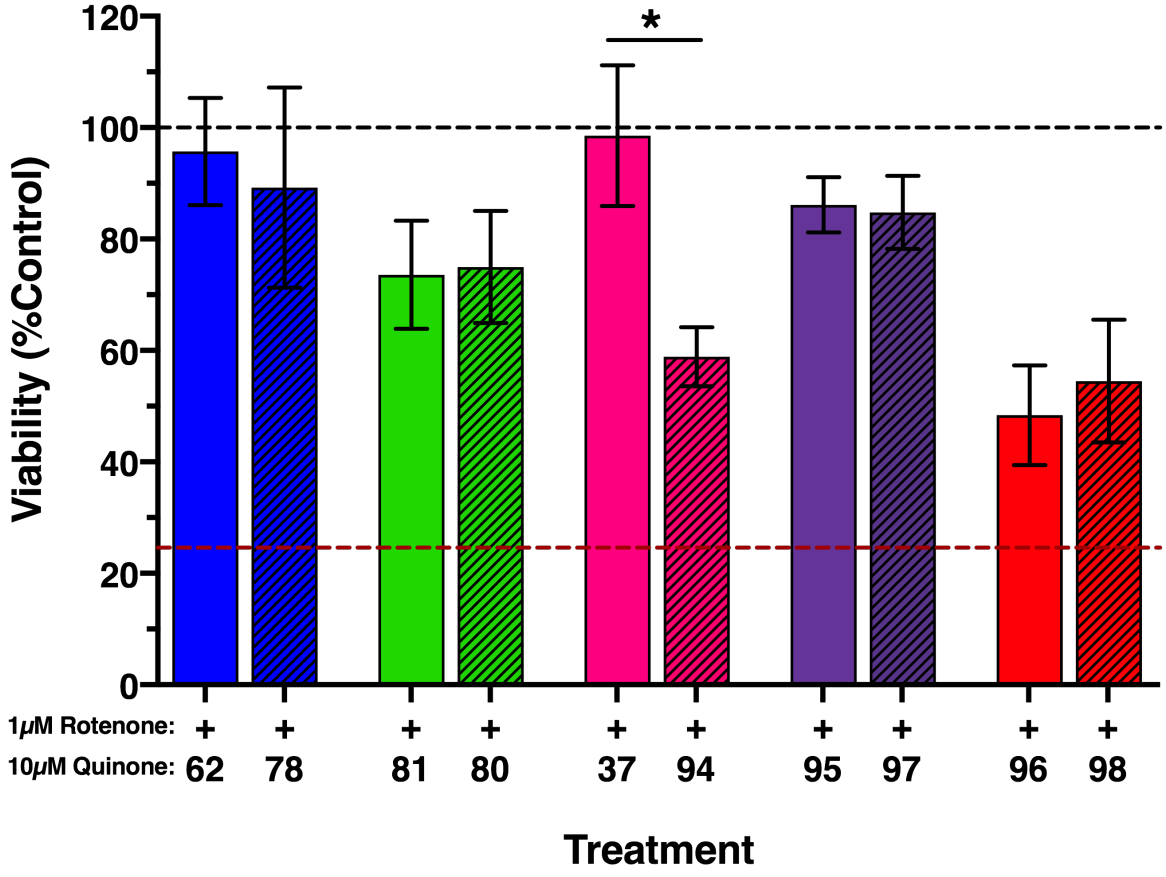


Figure 36: Effect of isomerism of the functional group on cytoprotection. Cytoprotection by the five pairs of L- (solid bar) and D-isomers (striped bar) of the test compounds (10 μM) in the presence of rotenone (1 μM) was expressed as percentage of sham-treated HepG2 cells (no rotenone, black dotted line). Each bar represents the activity of one compound, data is expressed as mean ± S.D. (n=3 independent experiments with 6 replicates within each experiment). Significance was calculated by using Student *t*-test to compare cytoprotection (p^* <0.05).

Red dotted line represents the cytoprotection by rotenone-only treated cells.

2.5 Discussion

Mitochondrial disorders are typically associated with defects in OXPHOS, reduced ATP levels, increased oxidative stress and decreased cellular viability. Under these conditions, several small-molecules such as SCQs are currently under clinical development to treat mitochondrial dysfunction or just alleviate disease symptoms (see section 1.3). A limited number of studies are published, justifying the effectiveness of SCQ molecules like idebenone (Klopstock et al. 2011, Lynch et al. 2010) and EPI-743 (Chicani et al. 2013, Zesiewicz et al. 2016) in a clinical setting. Currently, idebenone is the only marketed SCQ in Europe to specifically treat a mitochondrial disease, Leber's Hereditary Optic Neuropathy (LHON). SCQs are generally believed to protect against mitochondrial dysfunction by rescuing ATP levels and ameliorating oxidative stress by acting as antioxidants (Anisimov et al. 2011, Erb et al. 2012, Gueven et al. 2017, Krylova et al. 2016, Smith and Murphy 2010). However, there is some doubt around this simplistic view and their precise mechanism(s) of action of these compounds are yet to be understood and more importantly proven.

The main goal of the present study was to develop and characterise improved novel SCQ molecules, while working towards a better understanding of the underlying mechanism of action of these SCQs for treating mitochondrial dysfunction. In the current chapter, I have presented the data of the *in vitro* characterisation of those novel SCQs with regards to several cellular parameters that appear relevant in the context of mitochondrial dysfunction. However, I have to acknowledge that this list of endpoints is likely incomplete, and a multitude of additional endpoints could be argued for (Discussed in

detail in section 2.5.4). The selection of these parameters tested in this study was informed by the current (published) understanding of the molecular activities of SCS.

2.5.1 The effect of novel SCQs on protecting cell viability and rescue ATP levels in the presence of mitochondrial dysfunction

Mitochondrial dysfunction is typically associated with OXPHOS defects that result in reduced mitochondrial ATP production. Chronic mitochondrial dysfunction can lead to cell death. This is evident from my results, where the presence of the mitochondrial C-I inhibitor, rotenone, significantly reduced cellular viability to less than 30 % of sham-treated cells (Figure 19). Therefore, to develop novel drugs to treat mitochondrial dysfunction, it is important to investigate the efficacy of these compounds to protect cellular viability under defined conditions of mitochondrial dysfunction.

Although mitochondrial cybrid cells are widely used, there are some limitations associated with this approach. For example, a consistent reduction of cellular ATP levels could not be demonstrated in LHON cybrids under basal conditions (Baracca et al. 2005, Beretta et al. 2004), which is in contrast to peripheral blood mononuclear cells from LHON patients (Korsten et al. 2010). In addition, the exact patho-mechanisms of diseases associated with mitochondrial dysfunction are not clearly known at present. And while the same mtDNA mutation can give rise to different phenotypes, the same phenotype can be the consequence of different mutations (Blakely et al. 2005, Taylor and Turnbull 2005, Wallace 1992), which illustrates that a multitude of factors such as mutations in nDNA and mtDNA as well as nuclear polymorphisms, mitochondrial haplotype, epigenetic changes in tumor cells, and environmental factors can all affect disease symptoms and

etiology. These limitations make it difficult to generate a cell culture model of a specific mitochondrial disease that shows sufficient face-, construct- and predictive validity (Gazdar et al. 2010, Gómez-Durán et al. 2010). To prevent these limitations, I used a widely used hepatic cell line combined with a well-known mitochondrial toxin to generate a reliable model of mitochondrial dysfunction that could be reliably used over extended periods of time to perform the comparative studies of this chapter. Since quinones are known to undergo first-pass metabolism (Becker et al. 2010, Bodmer et al. 2009), I have chosen the hepatic cell line, HepG2 to characterise our novel SCQs. In addition, HepG2 cells are also known to express high levels of NQO1 (Wilkening et al. 2003). Inhibition of mitochondrial C-I with rotenone induces ROS-mediated toxicity that leads to apoptosis (Li et al. 2002). However, I have to acknowledge that this approach also had some obvious limitations Firstly, as the liver is known for detoxifying various metabolites, it would have significantly different defense mechanisms and tolerance to oxidative stress that may not be replicated by other cells (Bando et al. 2005). Moreover, due to the high basal antioxidant capacity of liver cells, it may also be difficult to assess the antioxidant capacity if our novel SCQs. Secondly, HepG2 cells, being cancerous in origin are known to have very high glycolytic capacity (Marroquin et al. 2007) and high levels of the enzyme NQO1 compared to normal liver which could over-emphasize results with regards to bioactivation and metabolic activity (Cresteil and Jaiswal 1991). While majority of NQO1 is localized in the cytosol, it is also found in the mitochondria of several tissues (Ellund et al., 1982; Ernster et al., 1962). Mitochondrial NQO1 is localized in the matrix and was reported to contribute to almost 15 % of the total cellular activity (Ellund et al., 1982; Ernster et al., 1962). Apart from NQO1, KGDHC in the mitochondrial matrix can also exhibit diaphorase-like activity (Klyachko et al., 2005; Kiss et al., 2014). The presence of these reductases in the mitochondrial matrix can also affect the activity of

quinone compounds by mediating NAD+ regeneration during C-I inhibition, hence supporting ATP generation via substrate level phosphorylation.

Lastly, in this study, I only have used a single mitochondrial C-I inhibitor, rotenone to induce mitochondrial dysfunction, similar to previously published study to test idebenone analogues (Erb et al. 2012, Giorgio et al. 2012, Haefeli et al. 2011). Idebenone is believed to rescue ATP levels by bypassing mitochondrial C-I, in the presence of rotenone-induced mitochondrial dysfunction. However, idebenone could not rescue ATP levels in the presence of mitochondrial dysfunction induced by other toxins such as antimycin or oligomycin that either inhibit mitochondrial C-III or block the proton pump of ATP synthase respectively (Erb et al. 2012). This replicates prior studies where menadione and CoQ1 in hepatocytes (Chan et al., 2002) and lymphocytes (Dedukhova et al., 1986,) inhibited the cytotoxicity caused by C-I inhibition, but not that caused by the inhibition of C-III (Kiss et al., 2014). Since our novel SCQs belong to the same chemical class as idebenone, we hypothesized that they would work by a similar mechanism. However, this approach limits our results by not being able to extrapolate them to mitochondrial dysfunction induced by other toxins or even other respiratory complexes. The results described in this study are therefore indicative to the effects of the novel SCQs on rotenone-treated HepG2 cells in a comparative manner only.

To measure the cytoprotective activity of SCQs, mitochondrial dysfunction was induced by treating cells with C-I inhibitor, rotenone (1 μM). Under these conditions, co-treatment with idebenone, increased cell viability from less than 30 % to more than 60 %. Simply changing the core structure of idebenone from a benzoquinone to a naphthoquinone (#2), increased cell viability to more than 60 %, suggesting that replacing the quinone core from benzoquinone to a naphthoquinone did not increase toxicity, while retaining the

cytoprotective potential of idebenone (Figure 19). The novel SCQs synthesised in this project are structurally similar to VK only based on the presence of a naphthoquinone core but display a much higher solubility due to a shorter side-chain that furthermore incorporates solubilizing groups. In contrast to benzoquinones, naphthoquinones are bioactivated not only by NQO1, but also by additional enzymes such as VK oxido-reductase isoenzymes (VKORCS1, VKORL2) (Tie and Stafford 2008, Wallin et al. 2008). Based on the ability to be bioactivated by additional enzymes, naphthoquinones should be associated less ROS-induced toxicity compared to benzoquinones in the absence of NQO1. Moreover, VK was found to be necessary to transfer electron in the mitochondria of *Drosophila* resulting in efficient ATP production, hence rescuing *Pink1* deficiency (Vos et al. 2012). However, VK-1, when tested for cytoprotection in the presence of rotenone, was toxic to the cells at 10 μM and reduced the cell viability to levels lower than cells treated with rotenone-only. As a transition to the novel SCQ library, testing the naphthoquinone core on its own, without any functional groups attached also (i.e. #23), did not protect cell viability any better than rotenone-treated cells. However, introducing a functional group at the 4C chain attached to naphthoquinone core (like #24), significantly improved cellular viability in the presence of rotenone (> 70 %), when compared to idebenone (Figure 19). These results suggest that it is not just the naphthoquinone core or any naphthoquinone molecule with long side-chains, but a combination of both the naphthoquinone core with selected functional groups attached to a side chain contribute towards the cytoprotective activity of the compound. Overall, more than 20 novel SCQs showed significantly better cytoprotection compared to the marketed idebenone (Figure 19, pink data points). In fact, some novel SCQs like #37 and #62 were not only significantly better in protecting cellular viability compared to idebenone, but fully restored viability back to non-rotenone treated. From these results,

it is evident that novel SCQs, with naphthoquinone core and additional functional groups on the side-chain can significantly protect cellular viability in the presence of mitochondrial dysfunction. However, these results do not provide any further information how these compounds work at the molecular level.

Mitochondria are involved in a multitude of cellular functions ranging from supplying chemical energy in the form of ATP and other high, energy-rich molecules such as NADH, Ca^{2+}-signaling, production of many metabolites and also ROS to the regulation of cell death (Davis and Williams 2012). Nevertheless, one of the main functions is ATP production (Vo and Palsson 2007). The reduced form of SCQ was reported to maintain ATP generation, in the presence of mitochondrial dysfunction by bypassing mitochondrial C-I (Erb et al. 2012). Once the electrons are donated to C-III, some re-oxidised quinones can shuttle out of the mitochondria to be reduced again by cellular reductases like NQO1, re-enter the cell and donate more electrons to C-III (Erb et al. 2012). This process is believed to act as a continuous shuttle in and out of the mitochondria, donating electrons and hence rescuing cellular ATP levels in the presence of mitochondrial dysfunction (Erb et al. 2012). Consistent with these finding, the authors suggest that the continuous shutting of SCQ requires a balanced solubility that facilitates membrane permeability. However, in contrast to their findings, neither a correlation between ATP rescue and solubility of the compounds, nor a narrow range of solubility was observed with our SCQs. This suggests that SCQs may promote any other compensatory mechanism within the mitochondria to maintain energy homeostasis (Baracca et al. 2005, Beretta et al. 2004).

If an SCQ can restore cell viability to 100 % under conditions of mitochondrial dysfunction, it is conceivable that this could correlate to its ability to restore cellular ATP levels. This notion is supported by the observation that the SCQ idebenone can rescue ATP levels under conditions of mitochondrial C-I while also protecting cell viability (Erb et al. 2012, Geromel et al. 2002, Gueven et al. 2017, Haefeli et al. 2011, Yu-Wai-Man, et al. 2017). However, the results from the present study indicate that for a large number of SCQs, their cytoprotective capacity does not correlate with their ability to rescue ATP levels (Figure 30). This is the first time that this hypothesis was tested and my results clearly question this long-held assumption. In fact, a consistent reduction of cellular ATP levels could not be demonstrated in LHON cybrids (Korsten et al. 2010). Previous *in vitro* and *ex vivo* studies also reported no reduction in ATP levels suggesting that cells may compensate the loss of ATP levels by upregulating other pathways for energy generation in the presence of mitochondrial dysfunction (Baracca et al. 2005, Beretta et al. 2004, Pommer et al. 2008). Therefore, the question of the molecular mode of action of SCQs remains wide open.

As a first step for a drug molecule to show its efficacy *in vitro*, it has to be able to enter the cells by crossing the plasma membrane, a function that is significantly affected by the solubility of the molecule (measured as logP, logD). A fine balance between both hydrophilicity and lipophilicity of the molecule is necessary for its entry into the cells, which can influence the efficacy of the molecule if the target is intracellular. A previous study with a panel of benzoquinones reported that the logD of the molecule, rather than its structural features, strongly determined its ability to rescue ATP levels under conditions of mitochondrial dysfunction (Erb et al. 2012). In contrast to this study, my

results did not demonstrate a strong association between cytoprotection and solubility (Figure 29). Compounds that were at least as protective as idebenone fell into a solubility range from log P = 1 to 7 and in contrast to the previous study with benzoquinones (Erb et al. 2012), no correlation or a narrow solubility range could be identified (Figure 29). The broad solubility range observed in the current study therefore only partially supports another study that suggested a log P < 5 for naphthoquinones to protect against neuronal cell death due to oxidative stress and mitochondrial dysfunction (Josey et al. 2013). Although there was no obvious correlation between cytoprotective ability and solubility of the compounds, a complex interplay of both solubility of the molecule along with other structural aspects (i.e. quinone core, length of the side chain and the type of functional groups attached to the quinone core) that result in specific electron density around the molecule, may allow some compounds to repeatedly shuttle through the mitochondrial membrane to rescue ATP levels.

2.5.2 SCQs can be bioactivated by several cellular reductases

Once SCQs have entered the cytoplasm, they are believed to be bioactivated by cellular reductases such as NQO1 to the active, hydroquinone form while simultaneously producing NAD^+ from NADH (Haefeli et al. 2011). In the absence of these 2-electron-transferring reductases, SCQs are not only ineffective as a cytoprotectants, but can also become toxic due to semiquinone formation that results in oxidative stress (Erb et al. 2012, Ishihara et al. 2012, O'brien 1991). In contrast to the semiquinone, some fully reduced hydroquinones can directly donate electrons to C-III of the mitochondrial ETC. Under conditions of C-I dysfunction, this mechanism is particularly favorable, since it maintains electron flow across the ETC and enables mitochondrial ATP synthesis. For

SCQs such idebenone, this C-I bypass was reported to be dependent on NQO1 (Giorgio et al. 2012, Haefeli et al. 2011). In agreement with previous reports, the majority of our novel SCQs depended on NQO1 for their reduction to the hydroquinone form (Figure 22). However, for the first time, we demonstrate that in our compound library, around 20 % of our compounds do not depend on dicoumarol-sensitive enzymes (such as NQO1) for their reduction to the hydroquinone form (Figure 22). Since our novel SCQs are naphthoquinones that show structural similarity to VK, it is conceivable that they could be bioactivated by the family of VK reductases. In fact, dependence of quinones solely on NQO1 for their bio-activation may be a matter of concern. Inactivating NQO1 polymorphism, are relatively frequent in the general population, with some ethnic groups such as the Chinese showing more than 70 % carriers for a single inactivating polymorphism (C609T) (Chhetri et al. 2018). In these individuals SCQs are likely converted to a larger extent to the highly reactive semi-quinone, which would induce ROS-dependent cellular damage (Gueven et al. 2015, Jaber and Polster 2015, Lewis et al. 2005). I hypothesized that, since some of our SCQs are reduced by reductases other than NQO1 (Figure 22), this should reduce their toxicity compared to idebenone. This is supported by the IC50 data of our SCQs which were at least 200 % higher in high reducing capacity cell line, HepG2, when compared to low reducing cell line, RGC5 (Conducted by another Master's student, data not shown). However, given the large number of compounds to be characterised and a general difficulty to assess *in vitro* toxicity of quinones due to their interference with many standard viability assays (Discussed in Chapter 4, Section 4.2), it was not possible to conduct a detailed investigation into the intrinsic toxicity for all compounds. This omission has to be noted as a possible limitation of the present study as is currently addressed in detail by another PhD student (Zikai Feng). However, the extent of chemical reduction of SCQs by cellular reductases did not

correlate with the measured cytoprotective or ATP rescue activities of our new SCQs (Figure 31). Therefore, our data suggests that enzyme-dependent reduction of quinones may not actually affect their cytoprotective abilities, which is a surprising finding and quite contrary to our common understanding of this class of compounds.

2.5.3 Influence of antioxidant activity on cytoprotection

Apart from reduction and rescuing ATP levels, quinones like CoQ_{10} and idebenone are largely believed to combat free radicals and hence prevent lipid peroxidation (Erb et al. 2012, Giorgio et al. 2012, Mordente et al. 1998). In contrast to this widely reported view and previous reports that supports this association for benzoquinones (Erb et al. 2012, Rahn et al. 2014), none of the novel SCQs in the present study significantly affected basal levels of lipid peroxidation. Additionally, as the effect of novel SCQs on BLP levels did not correlate to their cytoprotective ability (Figure 32), for the first time we propose that quinones are not just antioxidants, but they may be involved in a more complex mechanism to protect against mitochondrial dysfunction. However, we have to acknowledge that the HepG2 cell line used for these experiments might not be ideally suited to detect antioxidant effects. HepG2 cells are human liver-derived cells that still possess a large amount of their tissue heritage with regards to the detoxification of xenobiotics (Guillouzo et al. 2007). It is therefore not surprising that this cell line possesses significant endogenous antioxidative defenses (Bando et al. 2005) that might make it impossible to detect any SCQ-induced antioxidant effects. However, my results and interpretation are still valid since we observed SCQ-induced cytoprotection in the same cell line, irrespective of their endogenous antioxidant potential. This supports my

interpretation that antioxidative function appears to be irrelevant for cytoprotection by our SCQs.

2.5.4 Effect of SCQs on different energy generating pathways

To get a deeper insight into the mechanism of action of the novel SCQs, I characterised them against some major parameters that are likely to be influenced by mitochondrial dysfunction, such as lactate and ketone levels. Mitochondrial dysfunction forces cells to meet energy demands by utilising alternative mechanisms, such as increasing the rate of glycolysis. Increased glycolysis requires increased levels of NAD^+, that are needed for the central step of glycolysis, the conversion of glyeraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Typically, this NAD^+ is supplied by the conversion of pyruvate to lactate (Figure 8 from Chapter 1). Although, the production of lactate during glycolysis is generally seen as a cytotoxic byproduct of this process (Bodmer et al. 2008, Brewer 1998, Davidson et al. 1981, Dienel and Hertz 2001, Kaufmann et al. 2004, Walker et al. 2009) and a "dead-end" reaction, recent evidence suggests that cells from isolated rat liver, cardiac and skeletal muscle oxidise pyruvate and lactate for generating energy (Brooks et al. 1999, Brooks 2016). This is supported by an abundance of lactate oxidation complex in rat-derived muscle cells (Hashimoto et al. 2006). In fact, lactate was described as a preferred energy source in primary neuronal cultures (Pellerin 2003) and *in vivo,* in glial cells in *Cox10* (protoheme IX farnesyltransferase) mutant mice (Feron 2009, Fünfschilling et al. 2012).

Since the reduction of SCQs by cellular reductases is also accompanied by the production of NAD^+ from NADH, this process, at least in theory, could replace the cellular need for

lactate production. If this scenario could be induced by SCQs, the accumulation of lactate in cellular supernatant should be reduced. This hypothesis is supported by idebenone-induced reduced lactate levels in cybrids from MELAS patients (Haefeli et al. 2011). However, another SCQ, EPI-743 did not influence lactate levels in the blood of LS patients, after 6 months of treatment (Pastore et al. 2013). Supporting the current evidence, n this study, despite a general reduction of all novel SCQs in cells, only some of the SCQs with amino acids attached to the alkyl side-chain significantly decreased lactate levels in the present study, while SCQs with aliphatic ester attached to the alkyl side-chain significantly increased lactate levels in the cell culture supernatant (Figure 25). In addition, no obvious correlation between protection of cellular viability and the influence of SCQ on lactate levels could be demonstrated (Figure 33). Despite an upregulation of lactate levels by 175 % by SCQs, some of these compounds still protected viability up to 60 %, whilst the same was toxic for other SCQs. Even the compounds that showed no changes on lactate levels compared to sham-treated cells, protected viability ranging from more than 90 % to less than 30 % of sham treated cells. These results strongly suggest that any effect by an SCQ on the accumulation of lactate in cell culture supernatant is independent of the SCQs ability to protect against cellular viability.

An alternate source of cellular energy is the use of fatty acids via β-oxidation (Figure 5) (Nelson et al. 2008). While this process happens in most cells to different degrees, some tissues such as cardiac tissue satisfy their energy demands near exclusively from fatty acids (Lopaschuk et al. 2010, Watanabe et al. 2000). Fatty acids are a high-energy source but its use is associated with increased oxygen demands (Lopaschuk et al. 2010, McGarry and Foster 1980). A high-fat, low carbohydrate diet called ketogenic diet, recently gained a lot of attention due to its reported ability to protect against mitochondrial dysfunction (Lutas and Yellen 2013, Wang et al. 2018). Rodent studies showed that ketogenic diet

decreased oxidative stress and increased mitochondrial mass, associated with improved mitochondrial bioenergetics (Bough et al. 2006, Sullivan et al. 2004). Although the exact mechanism of how fatty acid oxidation combats mitochondrial dysfunction is not known, BHB decreased ROS production *in vitro* (Maalouf et al. 2007) and ketone bodies prevented ROS-induced cell death (Kim et al. 2007). In fact, 2 weeks of treatment with idebenone slightly increased ($p<0.05$) BHB levels in the blood of mice (Unpublished data, N. Gueven). The fact that five out of seven groups of novel SCQs increased basal ketone levels in cell culture supernatant upon treatment (Figure 27), could indicate that these SCQs may promote fatty oxidation as a source of energy. Although it is conceivable that the increased BHB levels could be cytoprotective in the test systems of the present study, the influence of SCQs on the extent of BHB accumulation in cell supernatant did not correlate with their cytoprotective effects (Figure 33).

Our results on the effect of SCQs on the accumulation of lactate or ketone levels in cellular supernatant suggest that the mechanism of action of these SCQs in protecting cellular viability is not as simple donating additional electrons to ETC or maintaining NAD^+ levels or upregulating BHB levels.

Overall, more than 20 novel SCQs were significantly more effective in protecting cellular viability compared to idebenone. I showed that novel SCQs can be bioactivated by other cellular reductases apart from NQO1, that they may act as anti-oxidants and alter lactate and BHB levels in cell culture supernatant (Table 7). However, since none of these parameters correlated with their cytoprotective activity, this strongly suggests that these individual factors are not related to the compounds' cytoprotective effects in the presence of a C-I dysfunction. Surprisingly, slight differences in the structures of the

molecules did significantly influence their cellular effects. Therefore, the exact mechanism of action of our cytoprotective SCQs remains unknown.

Table 7: Overview of the influence of SCQs on the various end-point assays

Functional group	Protection of viability (% Control)	Acute ATP Rescue (% Control)	Reduction by cellular enzymes (%)	Basal lipid peroxidation	Lactate levels	BHB levels
A	42.6 ± 23.7 **	49.2 ± 30.9 **	0.185 ± 0.194	0.93 ± 0.08	86.1 ± 26.7	2.5 ± 1.1 ###
B	36.3 ± 20.4	26.6 ± 27.9	0.053 ± 0.057	0.95 ± 0.06	79.4 ± 30.0	3.1 ± 4.6
C	49.9 ± 21.8 ***	51.7 ± 33.8 **	0.019 ±0.033	0.96 ± 0.03	72.0 ± 29.6	2.0 ± 0.5 ##
D	15.7 ± 3	15.1 ± 6.9	0.021 ± 0.016	0.95 ± 0.05	98.4 ± 23.2 #	1.8 ± 0.7
E	64.8 ± 8.2 ***	81.9 ± 12.6 ***	0.150 ± 0.115	0.94 ± 0.05	63.2 ± 13.6	2.0 ± 0.9 ##
F	69.7 ± 25.6 ***	59.4 ± 12.6 ***	0.031 ± 0.017	0.92 ± 0.06	55.3 ± 8.3 #	1.9 ± 0.4 #
G	76.5 ± 15.9 ***	84.3 ± 9 ***	0.223 ± 0.104	0.93 ± 0.07	59.8 ± 18.7	1.8 ± 0.6 #

A: Slight polarity; B: Aliphatic; C: Acid; D: Aliphatic ester; E: Amino ester; F: Amino acid; G: Amino alcohol. Each value represents mean ± S.D. for each SCQ (n=3 independent experiments with 6 replicates within each experiment. Student *t*-test to compare the cytoprotection by SCQs versus rotenone-only treated cells (p^{*} <0.05, p^{**}<0.01, p^{***}<0.001) and the effect of SCQs on lipid peroxidation, lactate and beta-hydroxy butyrate (BHB) levels against sham-treated (no SCQ) cells ($p^{\#}$ <0.05, $p^{\#\#}$<0.01, $p^{\#\#\#}$<0.001).

All endpoints tested in this study appear to be unrelated to the cytoprotective activity of the SCQs, which negatively affects the rational development of improved compounds. Unless new mode(s) of action or even protein targets are identified, progress in this field will be hampered by this problem. A recent patent might however provide a new lead (Cortopassi and Tomilov 2017). In this patent (patent number US 9,750,705 B2) the benzoquinone idebenone is described as a potent inhibitor of p52Shc. Shc is an intracellular signaling protein that is a part of a larger family of adaptor proteins that are involved in the differentiation and mitogenic signaling of a range of receptor kinases to downstream signaling entities. These receptors include the epidermal growth factor receptor (EGFR), nerve growth factor receptor (NGFR), platelet-derived growth factor receptor (PGDFR) and the insulin receptor. Upon activation of the receptor, p52Shc binds to the phosphorylated receptor leading a signaling cascade that ultimately lead to the inhibition of mitogen-activated protein kinase (MAPK) pathway, that leads to reduced proliferation (Ravichandran 2001, Thomas et al. 1995).

The data in the patent describe administering a p52Shc antagonist like idebenone for treating insulin sensitivity, impaired glucose tolerance or diabetes. Idebenone was claimed to significantly inhibit p52Shc activation at nanomolar concentrations and stimulate insulin sensitivity *in vitro* and *in vivo* in a dose dependent manner. Idebenone, administered to mice at 20 and 50 mg/kg PO was also claimed to increase insulin-dependent phospho-Akt and AMP-activated protein kinase (AMPK) activity in a dose-dependent manner. Akt activation is generally seen to induce a survival signal (Gerber et al. 1998, Wang et al. 2000) and AMPK activation typically leads to mitogenesis (Cortopassi and Tomilov 2017). Consistent with this model, mice with reduced p52Shc expression show increased brown-adipose tissue and an improved survival rate. There are also unpublished observations that idebenone can increase mitochondrial mass and

reduce proliferation in primary human muscle cells (N. Gueven, unpublished observation). Therefore, p52Shc appears to regulate cellular survival, mitogenesis, as well as mitogenic signals (Atwal et al. 2000, Besset et al. 2000, Nelson et al. 2003, Patrussi et al. 2005). The proposed model also suggests that inhibition of p52Shc protein may increase insulin sensitisation and also increase fatty acid oxidation. Intriguingly, in support of this model, insulin sensitisation by idebenone has been confirmed *in vivo* (N. Gueven, unpublished observation). However, despite this evidence, the exact p52Shc-dependent signaling mechanism that would protect against the rotenone-induced C-I dysfunction in our cell culture model remains unclear (Figure 37). It also has to be noted that these results are derived from a patent and have not been published yet. Therefore, they constitute non-peer-reviewed information that first has to be confirmed independently. Nevertheless, the fact that idebenone might significantly decrease p52Shc activation suggests that our novel structurally similar SCQs could have similar effects on Shc.

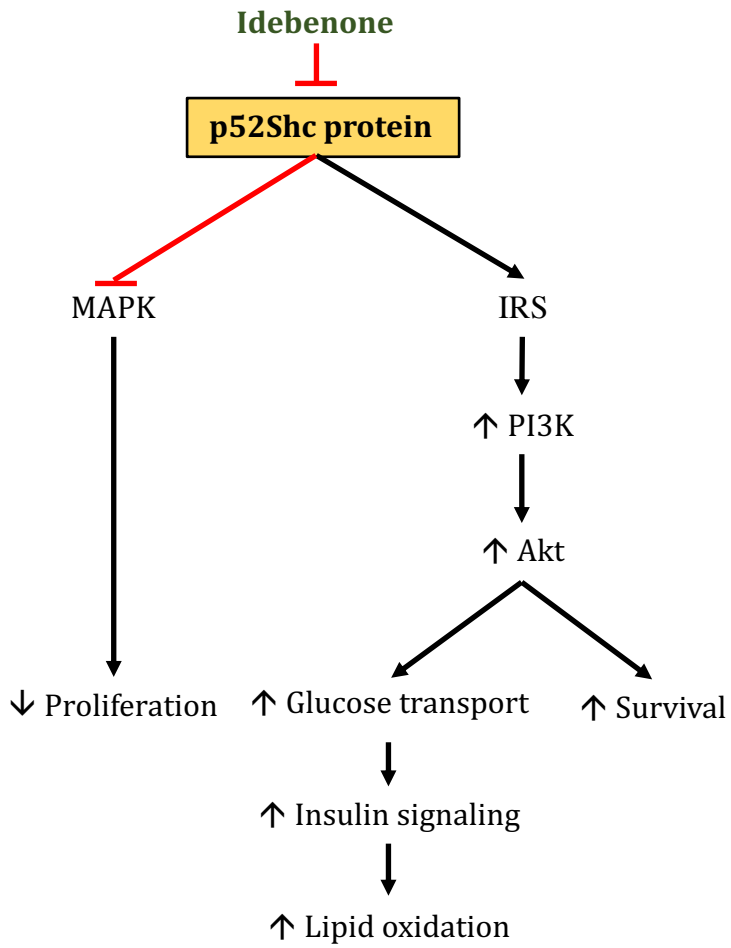


Figure 37: Effect of SCQ, idebenone on p52Shc. Idebenone inhibits the association of p52Shc with receptor tyrosine kinases, which in turn decreases mitogenesis and activates Akt-signaling thereby leading to an increased glucose metabolism and cellular survival.

IRS, insulin receptor substrates; MAPK, mitogen activated protein kinase; PI3K, phosphotidyl inositol-3-kinase.

Another potential mechanism of action of the novel SCQs was just recently described. In a mouse model of retinal-ischemic reperfusion (RIR) injury idebenone strongly induced Lin28A expression (Lei et al. 2018). Together with rasagiline, idebenone significantly restored retinal thickness and retinal ganglion cell survival, which was associated with restored visual function in these mice (Lei et al. 2018). This effect was dependent on the drug-induced upregulation of Lin28A. A potential mode of action that involves Lin28A is

an attractive possibility to explain the pleiotropic effects of this class of compounds since overexpression of Lin28A is reported to increase OXPHOS, insulin sensitivity, neurogenesis and tissue repair in general (Shyh-Chang et al. 2013, Zhu et al. 2011). It is also involved in glucose metabolism via the insulin-PI3K-mTOR pathway (Zhu et al. 2011) and the ageing process (Jun-Hao et al. 2016) but most importantly Lin28A defines stem-ness in several tissues as one of the key pluripotency markers (Shyh-Chang and Daley 2013). In another rodent model, overexpression of Lin28A in diabetic mice alleviated mitochondrial dysfunction, enhanced ATP levels, ETC complex activities and also inhibited the apoptosis of cardiomyocytes when compared to sham-diabetic mice (Sun et al. 2016). Therefore, since our novel SCQs, belonging to the same chemical-class as idebenone, they could also have a potential effect on Lin28A expression to improve cell viability in the presence of mitochondrial dysfunction. However upregulating Lin28A is a double-edged sword, since it is described to be an excellent marker for invasive malignant forms of a large range of cancers (Balzeau et al. 2017). Therefore, it would be surprising to see it associated with a drug such as idebenone with an excellent safety profile and no indication at all that it could induce or promote the development of malignancies in patients. Furthermore, Lin28A expression is generally described as promoting proliferation, which is in stark contrast to the effect of p52Shc-inhibition and our unpublished data showing reduced proliferation described above. Similar to the evidence for p52Shc inhibition, it has to be noted that the idebenone-Lin28A interaction was so far only described in a single paper and therefore has to be regarded with caution. Whether both of those activities described for idebenone can be extended to our SCQs and apply to other models and even tissues remain to be seen.

2.5.5 Structure-activity relationship of novel SCQs

Although the mito-protective properties of quinones have been studied for decades now, the structure-activity relationship (SAR) of quinones has not been evaluated in great detail based on the published literature. Using our novel SCQs, I worked towards establishing an SAR based on cytoprotection and acute rescue of ATP levels by SCQs in the presence of the mitochondrial C-I inhibitor, rotenone (Figure 30). Despite previous claims that quinones protect cell viability by rescuing ATP levels, our results indicated no obvious correlation between the two parameters (Figure 30). We initially started with SCQs that contained slightly polar, aliphatic, aliphatic esters and acid side-chain groups. These compounds generally showed a wide spread across the correlation plot (Figure 30). Although SCQs with an aliphatic ester side chain group tightly grouped in the correlation plot, they were neither cytoprotective nor could they rescue ATP levels. Based on these results, the next compounds were generated with increased polarity by adding an amino fragment at the end of the alkyl chain, which resulted in a noticeable trend in their activity by increasing both cell viability and ATP levels (Figure 30). Although there was no correlation between cytoprotection and acute rescue of ATP levels (Figure 30), addition of the amino ester significantly improved both cytoprotection and acute rescue of ATP levels, when compared to rotenone-only treated cells (Figure 19). These results formed the basis of generating the amino alcohol-containing compounds with intermediate polarity. Consistent with the previous results, SCQs with a terminal amino alcohol functional group on the side chain protected cell viability and also rescued ATP levels significantly better than rotenone-only treated cells (Figure 30). Overall, these results suggested that the polarity of the compounds is extremely important for their ability to protect cell viability and rescue ATP levels.

Quinones like CoQ_{10}, idebenone and MitoQ contain an alkyl side chain attached to ubiquinone core, while MitoQ and SkQ1 contain a terminal triphenyl phosphonium ion attached to alkyl side chain that is believed to target the molecule into mitochondria. The novel SCQs of this study were optimized by exclusively altering the functional group attached to the alkyl side-chain while containing the same naphthoquinone core. To understand the importance of the quinone core for the compounds cytoprotective activities, five of the best compounds (based on cytoprotection and ATP rescue) were selected where the naphthoquinone core was replaced with ubiquinone and plastoquinone cores respectively. Overall most compounds with a naphthoquinone core showed superior cytoprotection (Figure 34). These results suggest that the particular combinations of naphthoquinone core and specific functional side chain groups were responsible for the excellent cytoprotective ability of some of these compounds. To our knowledge, this is the first time that this direct comparison has been performed as most investigators typically only compare different side chains attached to a given quinone core (Erb et al. 2012, Rahn et al. 2014).

The next structural parameter studied was the importance of methyl group attached to the quinone core on position X (Figure 35). The majority of the currently clinically developed quinones including CoQ_{10}, idebenone, MitoQ and EPI743 contain the methyl group at position X, however this is absent in SkQ1 and some reported Vitamin K analogues (Josey et al. 2013). When the methyl group of the naphthoquinone core of five selected naphthoquinone was replaced with a hydrogen or hydroxyl group (Figure 35), in most of the cases, the compounds with methyl group showed significantly increased cytoprotection compared to compounds with hydrogen or hydroxyl groups. Chemically, it is conceivable that the presence of the methyl group could potentially prevent the metabolism of the naphthoquinone core. However, I must acknowledge that the SAR of

SCQs discussed so far would apply only to our novel naphthoquinones and cannot be extended to other quinones such as benzoquinones without additional experiments.

2.6 Conclusion

In this study, we successfully developed novel short-chain naphthoquinones to protect against mitochondrial dysfunction. For the first time, we could generate a SAR for the particular class of SCQs of the present study. The precise combination of a naphthoquinone core with a methyl group and an alkyl side chain with specific functional groups of balanced charge and polarity determined their cytoprotective activity. The developed SCQs protected cellular viability and rescued ATP levels in the presence of a mitochondrial C-I inhibitor, while influencing lactate and BHB levels in cell culture supernatant but not affecting basal lipid peroxidation. Importantly, none of the measured parameters correlated with the cytoprotective activities observed. Therefore, in contrast to the current, possibly over-simplified assumption that rescuing ATP levels and reducing oxidative stress are central to restoring cell viability, my results suggest that SCQs likely act in a more complex fashion that could involve recently described novel signaling pathways, including the signaling proteins like p52Shc or Lin 28A (Figure 38).

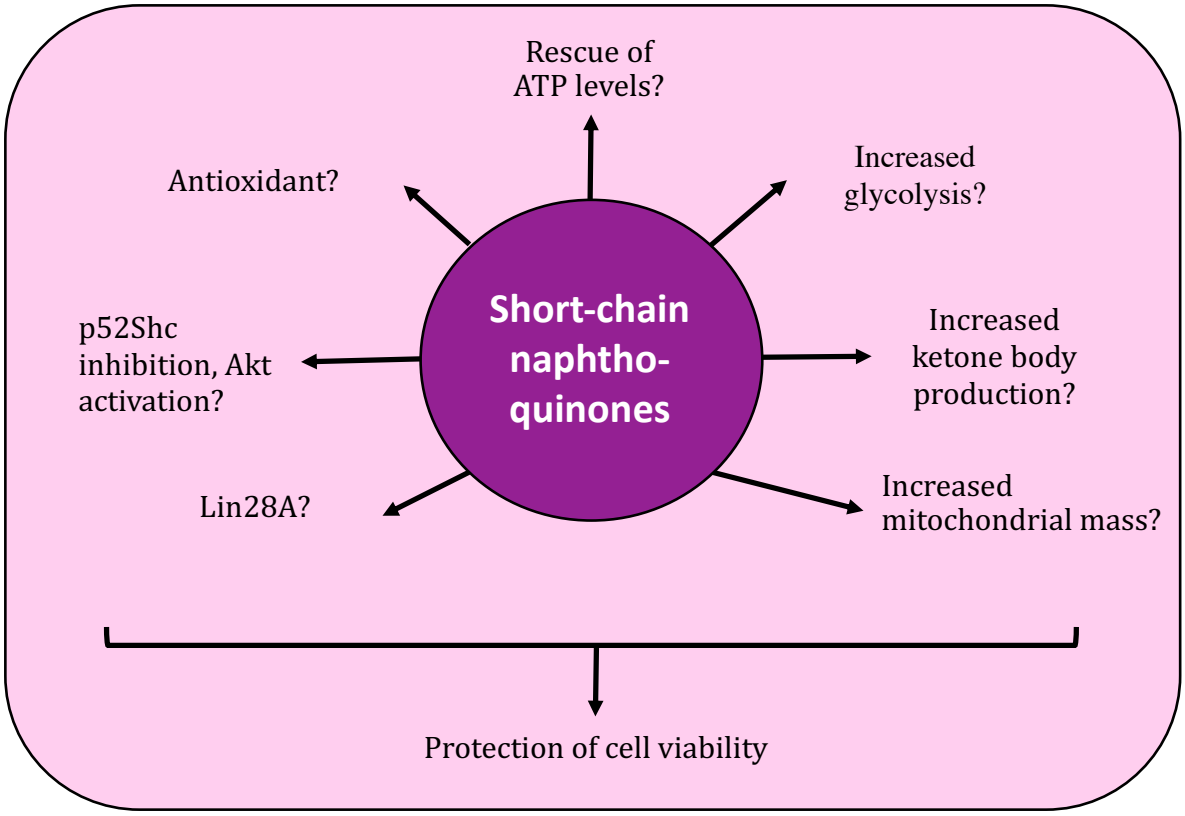


Figure 38: Influence of short-chain quinones on various metabolic, regulatory and signaling pathways that could potentially result in protecting cellular viability against mitochondrial dysfunction.

Chapter 3: Efficacy of selected short-chain quinones in a mouse model of mitochondrial dysfunction-induced blindness

3.1 Overview and rationale

The *in vitro* results described in the previous chapter indicate that selected novel short-chain quinones (SCQs) restore cellular viability *in vitro* to almost 100 % and rescue adenosine triphosphate (ATP) levels in the presence of the mitochondrial complex-I (C-I) inhibitor, rotenone. In order to translate my *in vitro* findings to an *in vivo* disease model, two selected novel SCQs were tested in a mouse model of Leber's hereditary optic neuropathy (LHON), where previously, the structurally related benzoquinone, idebenone significantly improved visual acuity (Heitz et al. 2012). This experimental model was particularly attractive, since blindness is induced by a single intraocular rotenone injection into only one eye. This approach does not lead to any obvious adverse off-target side effects, while visual acuity can be measured by a simple, non-invasive, behavioral read-out (Heitz et al. 2012). The cytoprotective ability of our novel SCQs along with positive results from the previously published study presented a strong rationale to progress some of our best SCQs to this *in vivo* disease model. Therefore, in this chapter, the efficacy of selected novel SCQs in this murine model of LHON will be discussed.

LHON is a rare inherited mitochondrial disorder, caused by mutations in genes that code for subunits of C-I of the mitochondrial electron transport chain (ETC). LHON is characterised by an acute loss of visual acuity and impaired colour vision (Yu-Wai-Man et al. 2011) (Detailed in section 1.2.1). While the vision loss in most patients is permanent, there is a limited chance of spontaneous vision recovery that can occur up to several years after disease onset in some patients (Spruijt et al. 2006, Stone et al. 1992). Therefore, it was hypothesized that the time interval between disease onset and spontaneous recovery could enable the restoration of visual acuity using pharmacological approaches

(Gueven 2014). Nevertheless, recent advances in our understanding of the disease pathology of LHON are yet to be translated in to safe and effective treatment options while only a very limited number of therapeutic approaches for LHON patients are currently in development (Table 10).

In pre-clinical studies, idebenone was shown to be a potent antioxidant, inhibited lipid peroxidation (Suno and Nagaoka 1984, Yu-Wai-Man et al. 2017) and rescued ATP levels by bypassing dysfunctional C-I in human hepatoma cells (HepG2), primary mouse hepatocytes (Erb et al. 2012, Haefeli et al. 2011) and primary fibroblast cell lines from LHON patients (Yu-Wai-Man et al. 2017) (Detailed in section 1.3). Based on these promising results that appear to overcome the central molecular defect of the molecular LHON pathology, idebenone was progressed to clinical trials. The first, phase 2, randomised, double-blind, placebo-controlled study (NCT00747487) ever performed in LHON patients included 85 patients with up to five years since the onset of vision loss (Klopstock et al. 2011). Of those, 55 patients, were treated with idebenone (900 mg/day; t.i.d.), while 30 patients received a corresponding placebo. The primary end-point of this study, best recovery of visual acuity from the baseline to 6 months, did not show statistically significant differences. This was not necessarily surprising given that some patients had been legally blind for several years and were likely in the chronic, atrophic state of the disease that is characterized by irreversible loss of retinal ganglion cells (RGC). However, a post-hoc responder analysis conducted for secondary end points suggested an increase in visual acuity in a subgroup of patients. Especially in patients that had only recently experienced a decline in visual acuity in line with the natural disease course, idebenone appeared to protect against further vision loss and in some cases significantly restored visual acuity when compared to placebo treated patients (Table 10) (Klopstock et al. 2011). A follow-up study showed that this protective effect of idebenone was

maintained for 30 months after the therapy had been terminated. Consistent with the previous report, a larger treatment effect was observed in patients where treatment was commenced within 1 year after the onset of disease (Klopstock et al. 2013). Idebenone also improved tritan colour vision in a randomised double-blind, placebo-controlled study on LHON patients (Rudolph et al. 2013). The effects were more profound in patients younger than 30 years and with less than one year since the onset of loss of colour vision (Rudolph et al. 2013).

In addition to idebenone several other approaches have been trialled in LHON so far. In contrast to idebenone, the Vitamin E-derived quinone EPI-743 did not show any significant improvements in visual acuity in small, open-label studies (detailed in section 1.3.4) (Enns et al. 2012, Sadun et al. 2012). In another small study, 6 out of 9 LHON patients (11778G>A mutation) showed significantly increased visual acuity and visual field size in response to a gene therapy approach that utilized adeno-associated virus to introduce an intact ND4 subunit of C-I (rAAV2-ND4) (Table 10) (Wan et al. 2016).

Other molecules such as cyclosporine and RP-103 have also been tested in LHON patients (Table 10), however, they did not result in any improvement in visual acuity (Leruez et al. 2018, Mancuso et al. 2010). Currently, there are five ongoing clinical trials in LHON, three using gene therapy, one using MTP-131 and another phase 4 trial with idebenone (Table 10).

Overall, the results from the few clinical trials, particularly from the trial using idebenone (NCT00747487), support the hypothesis that targeting mitochondrial dysfunction could in principle be beneficial to treat LHON patients but require more efficacious molecules and/or strategies.

Table 10: Therapeutic approaches in clinical trials to treat LHON (Status July 2018)

Treatment	Trial identifier	Study type	No. of subjects	Trial status	Outcome measurement	Result	Sponsor
Idebenone (900 mg/day)	NCT00747487	Phase 2, interventional, randomised, double-blind, placebo-controlled, parallel assignment	85	Completed	Primary end-point, recovery of visual acuity did not reach statistical significance. Colour contrast and retinal thickness improved significantly when compared to placebo (Klopstock et al. 2011).	Primary end-point not met, secondary end-point improved significantly	Santhera Pharmaceuticals
Curcumin (500 mg/day)	NCT00528151	Phase 3, interventional, randomised, double blind, placebo-controlled parallel assignment	70	Completed	Expected: Improvement in visual acuity.	N/A	Mahidol University
rAAV2-ND4	NCT01267422	Interventional, open label, single group assignment	9	Completed	No adverse events reported, 6 patients showed improvement in visual acuity (Wan et al. 2016).	End-point met	Huazhong University of Science and Technology
RP 103 (0.2-1.3 g/m^2/d)	NCT02023866	Phase 2, interventional, open-label, single group, dose-escalating study.	36	Completed	Significantly reduced oxidative stress but no significant improvement in clinical scale or quality of life (Mancuso et al. 2010).	End-point not met	Horizon Pharma USA, Inc.
KH 176 (dose N/A)	NCT02544217	Phase 1, interventional, randomised, double-	30	Completed	KH176 was well tolerated up to 800 mg/d, however significant changes in cardiac	End-point met	Khondrion BV

		blind, placebo-controlled dose escalating.			electrophysiology were observed at 2000 mg/d in healthy volunteers (Koene et al. 2017).		
Cyclosporine A (2.5 mg/kg/day)	NCT02176733	Interventional, open label, single group assignment	5	Completed	Oral cyclosporine did not prevent second-eye involvement (Leruez et al. 2018).	End-point not met	University Hospital, Angers
Idebenone (900 mg/day)	NCT01495715	Phase 3, interventional	N/A	Withdrawn	N/A	N/A	Santhera Pharmaceuticals
RP 103 (0.2-1.3 g/m^2/d)	NCT02473445	Phase 2, long-term, open-label extension study to assess safety, tolerability	22	Terminated	Terminated due to lack of efficacy in base study (NCT02023866)	End-point not met	Horizon Pharma USA, Inc.
Idebenone (dose N/A)	NCT02774005	Phase 4, interventional, open label, single group assignment	250	Ongoing	Expected: Safety and efficacy of long-term idebenone treatment	N/A	Santhera Pharmaceuticals
MTP 131 (1 % ophthalmic solution)	NCT02693119	Phase 2, interventional, randomised, double-blind, placebo-controlled, parallel assignment	12	Ongoing	Expected: Safety, tolerability and efficacy of MTP 131 in patients with LHON	N/A	Stealth BioTherapeutics Inc.
GS010	NCT02064569	Phase 1/2, interventional, open label, single group assignment	21	Ongoing	Expected: Safety and tolerability of GS010 in patients with LHON	N/A	GenSight Biologics

	NCT02652780	Phase 3, interventional, randomised, double blind, placebo-controlled, parallel assignment	36	Ongoing	Expected: Efficacy of GS010 for treating vision loss from 7 months to 1 year after LHON onset	N/A	
	NCT02652767	Phase 3, interventional, randomised, double blind, placebo-controlled, parallel assignment	36	Ongoing	Expected: Efficacy of GS010 for treating vision loss from 0-6 months after LHON onset	N/A	
rAAV2-ND4	NCT03428178	Interventional, open label, single group assignment	50	Ongoing	Expected: Efficacy of rAAV2-ND4 for treating vision loss within 3 months after LHON onset	N/A	Huazhong University of Science and Technology

N/A, not available.

3.2 Aims and objectives

Currently, only a few drug candidates and treatment strategies are in development that selectively aim to restore mitochondrial function. Therefore, in this chapter, two of the most promising novel SCQs, #37 and #77 that protected cellular viability *in vitro* to 100 % of sham-treated cells, while rescuing ATP levels, in the presence of the C-I inhibitor, rotenone, were tested in a mouse model of LHON. However, both compounds differ significantly in solubility. The SCQ #37 is a quinone with amino acid moiety as the terminal group that corresponds to a theoretical log P and log D of 3.42 and 0.12 respectively. This illustrates that solubility of #37 is highly pH dependent and therefore could affect absorption and tissue penetration. In contrast, the SCQ #77 which is a quinone with amino alcohol moiety as the terminal group has a log P and log D of 3.41. the SCQ #77 illustrates a significantly lower solubility that is less likely affected by tissue pH levels. However, a logD of 3.41 seems ideal for brain penetration and could therefore be superior with regards to absorption into the eye compared to #37. Therefore, in this chapter, I aimed to translate my *in vitro* results (Chapter 2) to an *in vivo* mitochondrial disease model and compare their efficacy to the currently used drug, idebenone.

To achieve this aim, in this chapter, experiments were conducted to fulfill the following objectives:

- To establish a previously described mouse model of drug-induced acute vision loss, in our lab environment
- To measure the influence of oral administered SCQs on the visual acuity of mice
- To analyse the effect of SCQs on the molecular pathology of the mouse retina

3.3 Materials and Methods

All reagents were sourced from Sigma Aldrich, Castle Hill, Australia unless otherwise specified.

3.3.1 Animals

Male, C57Bl/6 mice, aged between 8-11 weeks, with an average body weight of 25 g were used for the current study. C57Bl/6 mice were selected as they showed a robust optomotor response in a previously published behavioural study that demonstrated neuroprotection by idebenone (Heitz et al. 2012). Female animals were deliberately avoided in this study since the female sex hormone, oestrogen is suspected to exert some neuroprotective activity in the retina and was described to be cytoprotective *in vitro* in cells derived from LHON patients (Giordano et al. 2010). This hypothesis is supported by the very low numbers of female LHON patients who have been described in the literature (Man et al. 2002, Man et al. 2003). Therefore, I aimed to approach the real-life scenario by using young, male mice in this study. All procedures were performed in accordance with the Australian code for the use of animals for scientific purposes and under the required animal ethics approval from the University of Tasmania (UTAS) Animal Ethics Committee (Approval number A0016080, approval permit located in Appendix 3).

3.3.2 Transport and housing of mice

All mice were derived from stock bred at the UTAS Cambridge farm facility (CFF). Animals were transported in cages by vehicle from the CFF to the L6 animal holding facility at the UTAS Medical Sciences Precinct (MSP). Animals were given at least 7 days (d) to acclimatise to the MSP facility conditions to reduce stress induced by transportation and change in environment before they were used for the experiments.

All mice were orally supplied with test compounds by mixing them into individual daily food portions as previously described (Heitz et al. 2012) that allowed exact dosing for each animal. This approach was favoured over gavage application to avoid unnecessary stress, which was essential to generate meaningful behavioural data. To control drug intake, mice were individually caged and provided with a physically enriched environment that included housing and bedding material, small toys, autoclaved toilet rolls, small wooden sticks for gnawing and glass marbles to enable natural behaviour (Baumans 2005).

3.3.3 Randomisation of mice into treatment groups

To avoid bias, all mice were randomised into the four treatment groups: sham-treated, idebenone-treated, #37-treated and #77-treated by balancing the influence of baseline characteristics (covariates) of the mice, i.e. their parents and body weight as previously described (Suresh 2011, Hirst et al. 2014). Depending on the availability of mice from CFF, whenever possible (33 out of 40 mice in total), littermates were allocated into different treatment groups to ensure genetic complexity within each treatment group. The goal

was to achieve an average body weight of approximately 25 g and an average age of 10-weeks within each group of 10 mice before the start of the experiment. Since handling all 40 mice at once would have impaired reliable behavioural data (as behaviour was only assessed between 8 - 10 am to avoid circadian influences), mice were requested in five separate batches at different time points. To avoid batch-to-batch variation, mice within individual batches were allocated to all 4-treatment groups, where possible (for details of individual mouse allocations see Appendix 4, Table A17-A20).

3.3.4 Food intake experiments

Since our novel SCQs were significantly better in protecting cellular viability and rescuing ATP levels *in vitro* compared to idebenone, I treated the mice with a 10-fold lower dose (200 mg/kg) than previously published for idebenone (2000 mg/kg) in the same model (Heitz et al. 2012). All mice from the four treatment groups were pre-treated with the respective SCQs or sham-treated at 200 mg/kg idebenone molar dose equivalents every day for 7 d before exposure to the mitochondrial toxin, rotenone, and subsequently for the remainder of the study period. Equivalent dosage (mg/kg body weight) for the novel SCQs were calculated as equimolar doses based on 200 mg/kg idebenone and the specific molecular weights of the novel SCQs (Table 11). The equimolar dosages were calculated as 200 mg/kg, 240 mg/kg and 249 mg/kg for idebenone, #37 and #77 respectively (Table 11).

Table 11: Calculating equivalent dosage for novel short-chain quinones

Test compound	Molecular weight (g/mol)	Amount of test compound (mmol/kg)	Equivalent dose (mg/kg)
Idebenone	338.44	0.59095	200
37	405.44	0.59095	240
77	421.49	0.59095	249

SCQs were formulated with food powder (Speciality feeds, WA, Australia) to produce individual portions and were given once a day as previously published (Heitz et al. 2012). Briefly, SCQs were stirred overnight at 4 °C in 500 ml 0.5 % carboxymethylcellulose sodium salt (CMC) in water to generate a 20 mg/ml solution. Then 37.5 ml of the corresponding SCQ in CMC solution, 41.25 g sucrose, 371.25 g food powder and 0.5 % CMC solution to a final overall weight of 825g were mixed to prepare a food mash (Table 12). In the last step, the food mixture was aliquoted (5.5 g each) in small weighting trays and individually stored at –20 °C. Sham-treated animals received the same food portions excluding drug. The aliquoted food-SCQs portions were stable in the freezer for up to 3 years. All mice received one portion of the aliquoted food mixture per day (between 4 - 6 pm), corresponding to their treatment group allocation. Animals rapidly accepted this form of food supply and it was observed that this food was eaten preferentially, while *ad libitum* supply of standard food pellets (Speciality feeds, WA, Australia) and water was also ensured throughout the study. Idebenone powder was kindly donated by Santhera Pharmaceuticals (Muttenz, Switzerland) and the test compounds were provided by the Department of Chemistry (Dr. Krystel Woolley, UTAS) in sufficient quantities at a purity of >95 %.

Table 12: Recipe for preparing 150 portions of drug containing food mash

	Idebenone	**#37**	**#77**
Drug solution (20 mg/ml) in 0.5 % CMC* (ml)	37.5	44.9	46.70
Sucrose (g)	41.25	41.25	41.25
Food powder (g)	371.25	371.25	371.25
0.5% CMC (ml)	375	367.85	366.05
Total weight (g)	825	825	825
Individual portion (g)	5.5	5.5	5.5

*CMC, carboxymethylcellulose, sodium salt

Additional food intake in addition to the food mash was calculated from the pellet hopper weight and is presented as cumulative additional food intake. Body weight of mice and food intake was measured every 1 to 5 d. Relative food intake was calculated as the cumulative change in food intake (g) relative to cumulative change in body weight (g).

3.3.5 Intraocular Injections

To induce rotenone-induced blindness in the animals, mice were anaesthetised in a flow chamber with 5 % isoflurane and oxygen (600 ml/min) until sedated. The foot withdrawal reflex in response to foot pinch test was used to determine the extent of anaesthesia. Upon sedation, a nose-cone delivering 2 % isoflurane (300 ml/min using oxygen) was placed over the mouse's nose for the duration of surgery. The concentration and rate of anaesthetic delivery were increased slightly (up to a maximum of 5 %

isoflurane, 500 ml/min) depending on the respiratory response of the anesthetized mouse. Prior-to and after intraocular injection, cotton buds soaked in sterile saline were used to clean the area around the eyes. For intravitreal injection, a 31 -gauge needle was first used to puncture the sclera and the eye was gently massaged to remove a small amount of vitreous and to prevent increased intraocular pressure. Then, a 33 -gauge needle adapted to a 10 µl Hamilton syringe (Intraocular injection kit, World Precision Instruments, USA) was used to introduce 1 µl of rotenone (5 mM in dimethyl sulphoxide, DMSO) to the vitreous chamber of the left eye. The needle tip was inserted into the superior hemisphere of the eye, at the level of the pars plana and at a 45 ° angle through the sclera into the vitreous body. This route of administration avoids retinal detachment or injury to eye structures, including the lens and the iris. After the surgery, the mice were allowed to recover on a heating pad until they regained consciousness and then returned to their home cages.

The injections were performed blinded with regards to the treatment group allocation of individual mice. The injected eye was carefully checked once a day for 7 d post-surgery for any signs of inflammation.

3.3.6 Behavioural analysis (optomotor response)

To assess visual acuity in the animals, their optomotor response (OMR) was assessed as previously described (Heitz et al. 2012). For two weeks before and 10 weeks after rotenone injection, the OMR of the mice was repeatedly tested once every week over the entire duration of the experiment. For this purpose, the mice were individually placed on

a small platform surrounded by a motorized drum (30 cm diameter) with vertical black and white stripes (1 cm thick, Figure 39).

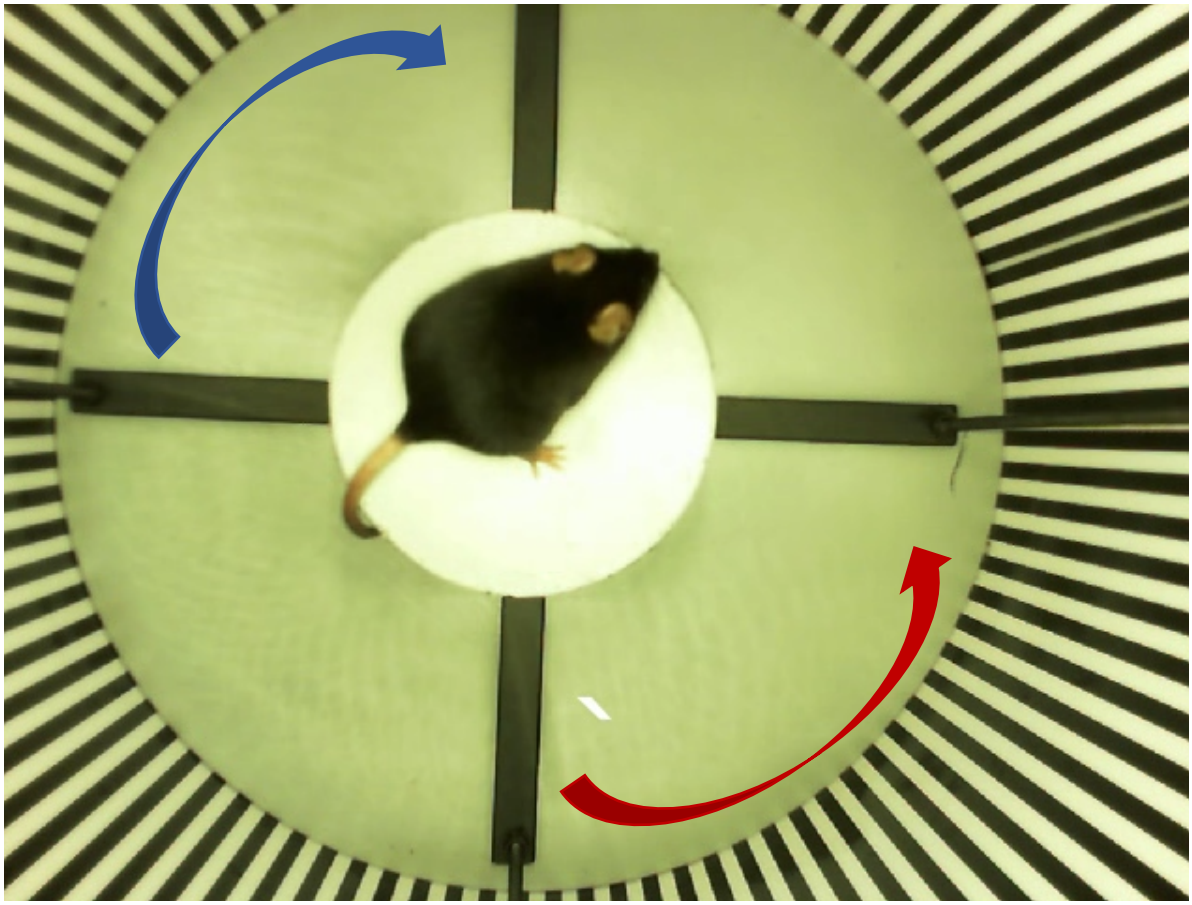


Figure 39: Experimental setup for measuring visual acuity of mice. Mice were placed on a stable platform in the centre. Visual acuity was quantified by scoring the number of head-turns while rotating the stripes of the optokinetic drum (with the centre stage remaining stable) for two min in clockwise (blue arrow, measures left eye function) and anti-clockwise (red arrow, measures right eye function) direction.

After 10 min of adaptation to the system, the visual acuity testing was performed by rotating the drum clock-wise and counter-clock-wise at two revolutions/min for 2 min in each direction and with an interval of 30 sec between the two rotations. Mouse behaviour was recorded with a digital video camera for subsequent scoring of head tracking movements as previously described (Heitz et al. 2012). All videos were completely de-

identified by a person not involved in the project prior to manual scoring to avoid investigator bias. After the full analysis of all videos, the data was decoded and the results analysed for the left (injected) and right (non-injected) eye and expressed as head movements.

3.3.7 Responder analysis

While vision loss in most LHON patients is permanent, there is a limited chance of spontaneous vision recovery that can either occur in the first year or up to several years after disease onset in some patients (Spruijt et al. 2006, Stone et al. 1992). This characteristic is replicated by the mouse model used in this study and is dependent on the amount of injected rotenone (unpublished observation N. Gueven). Therefore, to quantify the number of mice that regained visual acuity, either spontaneously or in response to SCQ-treatment, a responder analysis was performed. At the end of the observation period (71 d), the number of mice that showed more head-turns compared to sham-treated mice at 1 d post-rotenone injection were considered as responders and reported as “Responders (%)”. In a second assessment, the number of mice that showed more head-turns at the end of treatment period (71 d) than sham-treated mice at any given time during the treatment period were considered as responders to SCQ treatment and reported as “Responders to SCQ-treatment (%)”.

3.3.8 Euthanasia and cardiac perfusion

At the end of the observation period all mice were sacrificed for tissue analysis. Mice were terminally anaesthetised with intraperitoneal sodium pentobarbital (110 mg/kg body weight). For histology studies, mice were transcardially perfused with 4 % paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 1 to 2 min to clear the blood from the vasculature and effectively fix the tissues. Eyes were immediately collected and post-fixed in 4 % PFA in 0.1 M PBS overnight at 4 °C, before soaking in 30 % sucrose 0.1 M PBS for 24 h followed by 18 % sucrose in 0.1 M PBS for another 24 h at 4 °C. Tissue samples were stored in 0.1 M PBS with 0.02 % sodium azide until used for histology studies.

3.3.9 Histological analysis of mice eyes

3.3.9.1 Tissue processing

Eyes stored in PBS with 0.02 % sodium azide were processed using a tissue auto-processor (A82310100, Excelsior AS Tissue Processor, Thermo Fisher Scientific Pty Ltd, VIC, Australia). The tissue processing involved a series of steps that started with dehydration by an ethanol series, which was followed by clearing in xylene and subsequent infiltration with paraffin wax (Table 13).

Table 13: Steps for tissue processing.

Solution	Duration (min)	Temperature (°C)
Ethanol (70 %)	15	37
Ethanol (95 %)	30	37
Ethanol, absolute	15	37
Ethanol, absolute	15	37
Ethanol, absolute	15	37
Ethanol, absolute	20	37
Xylene	35	37
Xylene	35	37
Paraplast wax	60	60
Paraplast wax	60	60
Paraplast wax	60	60

3.3.9.2 Wax embedding of tissue and microtomy

Processed eyes were then embedded into paraffin blocks using stainless-steel moulds. A semi-automatic precision microtome (Leica RM2235, Leica Microsystems Pty Ltd, VIC, Australia) was initially adjusted to 10 µm for coarse sections until the area of interest was reached and then adjusted to 4 µm to cut thinner sections suitable for staining. The thin sections were mounted onto adherent glass slides (Dako, NSW, Australia) and dried for 15 min at 65 °C, before stored at room temperature until stained.

3.3.9.3 Haematoxylin and Eosin (H & E) staining

Paraffin sections on glass slides were dewaxed, rehydrated and H & E stained at room temperature using an auto-stainer (Leica Auto-stainer XL, Leica Microsystems Pty Ltd, VIC, Australia) (Table 14). After drying, the slides were mounted with mounting agent (Dako, NSW, Australia), followed by a coverslip (Fronine Pty Ltd, NSW, Australia) to seal. The stained slides were stored at room temperature until imaged using a light microscope (DM 2500, Leica Microsystems Pty Ltd, VIC, Australia).

Table 14: Steps for dewaxing, haematoxylin and eosin (H & E) staining paraffin-sectioned mice tissue.

Solution	Duration (sec)
Xylene	30
Xylene	30
Ethanol, absolute	30
Ethanol (95 %)	30
Ethanol (70 %)	30
Water wash	30
Haematoxylin	360
Water wash	30
Acid alcohol differentiator	20
Water wash	30
Ammoniated water	60

Water wash	30
Ethanol (70 %)	30
1 % Eosin phosphate staining solution	60
Ethanol, absolute	20
Ethanol, absolute	20
Ethanol, absolute	20
Xylene	20
Xylene	20

3.3.10 Analysis of stained sections

To generate comparable photographic representations of tissue samples, all images were acquired at 40 X magnification and at the same distance from the retinal periphery as suggested previously (Mead et al. 2014). Subsequently, the number of RGCs in the ganglion cell layer (GCL) were scored manually for the entire retinal image using ImageJ software and were reported as RGC/mm. The retinal thickness was calculated by measuring the distance from the innermost border of the GCL to the outermost border of the photoreceptor layer (PL) of the retina. Retinal thickness was measured at 3 separate points on each image, i.e. the left and right ends and the centre. The average of three measurements was calculated and reported as µm. Both RGC numbers and retinal thickness were calculated from an average of 7-11 animals per treatment group (n=7-11, one section per mice). Sections with obvious retinal damage due to procedural mistakes (i.e. cutting artefacts such as tissue folding and tearing) were excluded from the analysis.

All data are represented as mean or mean ± S.D. as denoted in the figure legends. Statistical analysis to measure the significance between different groups was performed using Student *t*-test (GraphPad prism software; GraphPad Software Inc., CA, USA).

3.4 Results

3.4.1 Revisiting *in vitro* data for idebenone, #37 and #77 from previous chapter

The novel SCQs #37 and #77 (Figure 40 ii, iii) were chosen to be tested in the LHON mouse model as both compounds restored cellular viability *in vitro* to almost 100 % in the presence of rotenone, which was significantly better than idebenone. In addition, both compounds differ significantly with regards to their physicochemical characteristics. The SCQ #37 is a quinone with amino acid moiety as the terminal group. This structure corresponds to a theoretical log P and log D of 3.42 and 0.12 respectively. This illustrates that solubility of #37 is highly pH dependent and therefore could affect absorption and tissue penetration. In contrast, the SCQ #77 which is a quinone with amino alcohol moiety as the terminal group has a log P and log D of 3.41. the SCQ #77 illustrates a significantly lower solubility that is less likely affected by tissue pH levels. However, a log D of 3.41 seems ideal for brain penetration and could therefore be superior with regards to absorption into the eye compared to #37. An additional point of difference between the two compounds was their effect on β-hydroxy butyrate (BHB) levels in cell culture supernatant. While the SCQ #37 significantly reduced BHB levels in cell culture supernatant, the SCQ #77 showed a trend towards increased BHB levels compared to idebenone (Figure 41 i). Furthermore, reduction of SCQ #37 by cellular reductases occurred at a significantly lower level compared to idebenone, while reduction of #77 occurred at a significantly higher level (Figure 41 ii). For all other assays, no significant differences between the novel SCQs and idebenone were observed (Figure 41 i). I hypothesized that the selective use of two compounds with the described observed *in*

vitro differences would enable me to assess the significance of those parameters for their protective efficacy *in vivo*.

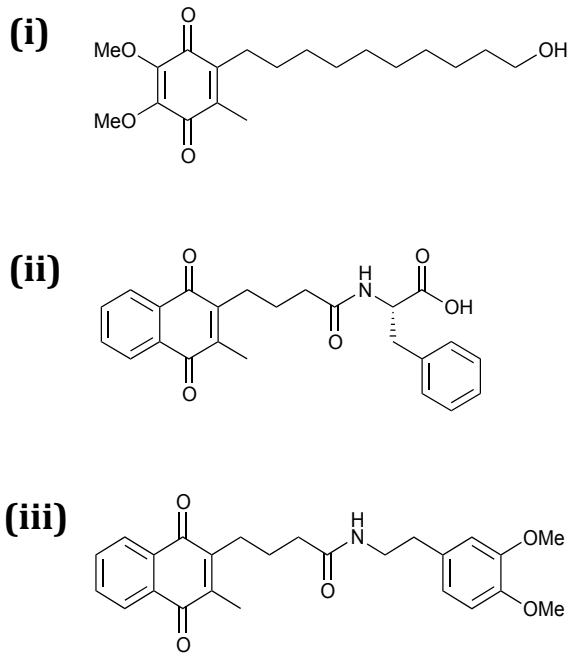


Figure 40: Molecular structures of (i) idebenone, (ii) novel short-chain naphthoquinones (SCQs) #37, (iii) novel SCQ #77.

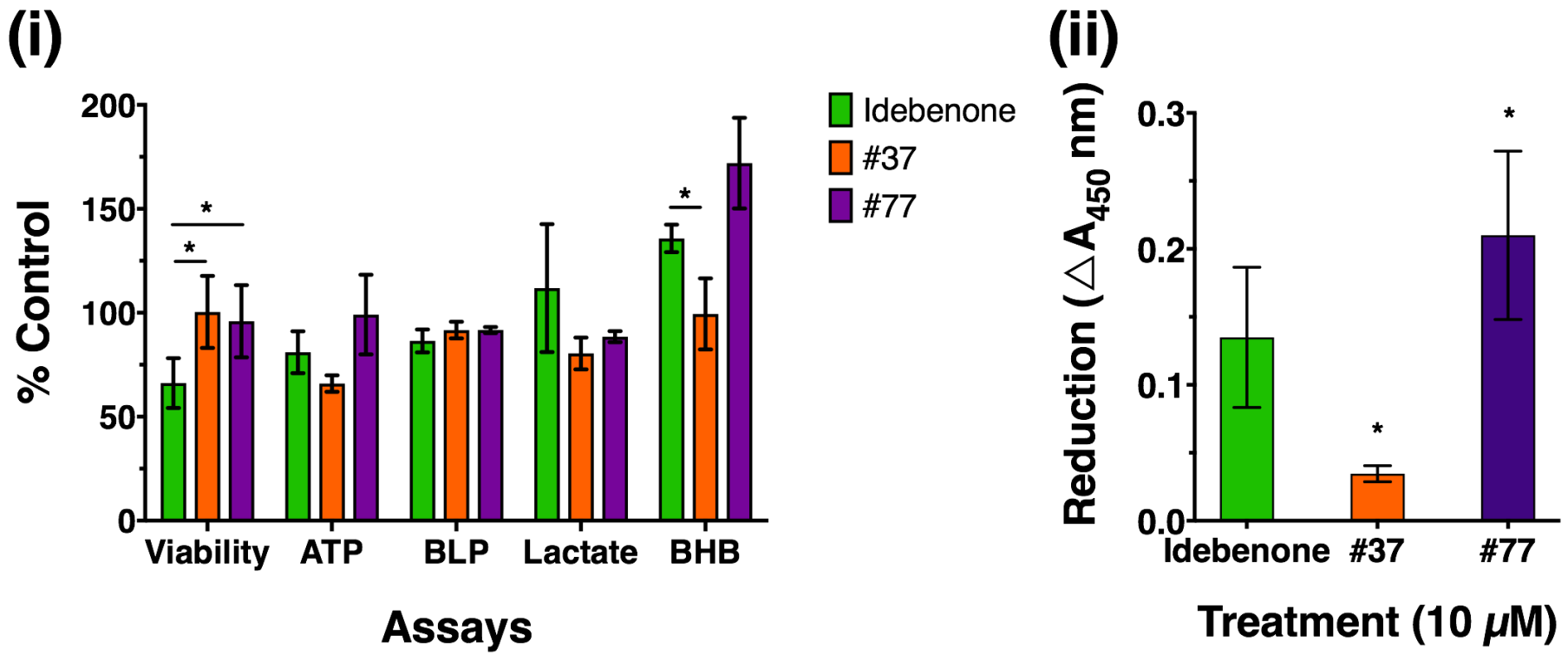


Figure 41: Summary of results from all *in vitro* characterisation assays for idebenone, novel short-chain quinones (SCQs) #37 and #77 (Summary of data from Chapter 2). (i) Effect of SCQ (10 μM) on cell viability in the presence of 1 μM rotenone, acute rescue of adenosine triphosphate (ATP) levels in the presence of 10 μM rotenone, basal lipid peroxidation (BLP) levels, lactate levels and β-hydroxy butyrate (BHB) levels calculated as percentage of sham-treated cells **(ii)** Reduction of SCQ (10 μM) by cellular reductases. Data is expressed as mean ± S.D. (n=3 independent experiments with 6 replicates within each experiment). Significance was calculated by using Student *t*-test to compare SCQ against idebenone using GraphPad Prism software ($p^* < 0.05$).

3.4.2 Randomisation of mice into treatment groups

Initially, to establish the model of rotenone-induced vision-loss at UTAS as previously reported (Heitz et al. 2012), the animals of the first batch of mice (n=8) were randomised into sham and idebenone treatment groups. A significant loss of visual acuity was observed in the left, rotenone injected eye one day after injection, while the visual acuity of right (un-injected) eye remained unchanged in all mice (Figure 49). Mice that did not show a significant loss of visual acuity after rotenone injection (n=2) were excluded from the study.

After confirming drug-induced vision loss, mice from the second batch onwards were randomly allocated to all four-treatment groups to achieve groups that contained mice of approximately 10-week of age with an average body weight of approximately 25 g (n=10 mice/group) (Appendix 4, Table A17-A20). The age of mice within individual treatment groups was 70.1 ± 6.0 d, 69.0 ± 1.9 d, 67.2 ± 6.3 d and 67.8 ± 6.7 d within sham, idebenone, #37 and #77 treatment groups respectively (Figure 42 i). The average body weights were 23.5 ± 1.8 g, 23.8 ± 1.9 g, 23.3 ± 1.1 g and 23.9 ± 1.0 g within sham, idebenone, #37 and #77 treatment groups respectively (Figure 42 ii).

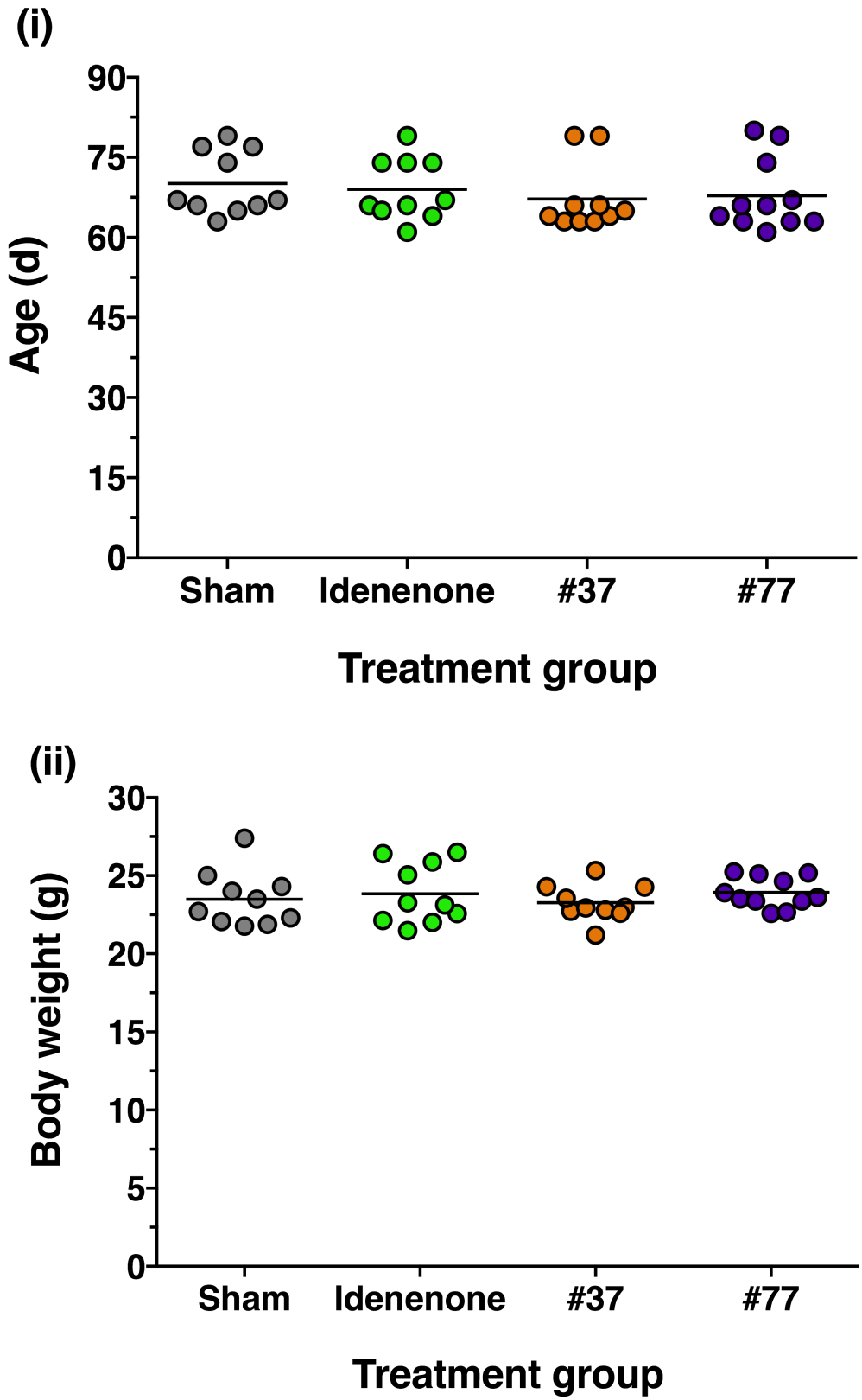


Figure 42: Allocation of mice to different treatment groups. Average **(i)** age (d) and **(ii)** body weight (g) of mice within each treatment group when the animals were included in the study. Each data point represents one mouse.

3.4.3 Influence of SCQ treatment on food intake and body weight of mice

Since the novel SCQs were tested for the first time *in vivo*, it was important to monitor for any SCQ-induced toxicity. For example, a 10 % decrease in body weight was considered as an adverse event, however such a decrease was not observed in any of the treated mice. Other adverse events such as changes to mice behaviour, fur condition, any signs of infection in the eye were also monitored routinely. No adverse events were observed in any experimental mice.

Relative food intake was calculated to quantify the influence of SCQs on food intake and body weight of the mice. For almost one-week post rotenone injection, the relative food intake was slightly variable within the treatment groups (Figure 43 ii). For example, at 1 d post rotenone injection, the relative food intake was 31.85 ± 80.91 for #77 treated group, while it was 27.55 ± 61.07 at 3 d for idebenone treatment group. These differences arose since in a few mice the cumulative increases in body weights were much lower when compared to the cumulative increase in food intake (Figure 43 ii). However, from the second week-post rotenone injection, the relative food intake was stable and relatively consistent across different treatment groups (Figure 43 ii). Overall, the average relative food intake was 6.02 ± 4.17, 8.41 ± 11.35, 4.72 ± 0.86 and 5.4 ± 12.9 for sham, idebenone, #37 and #77 treated mice respectively.

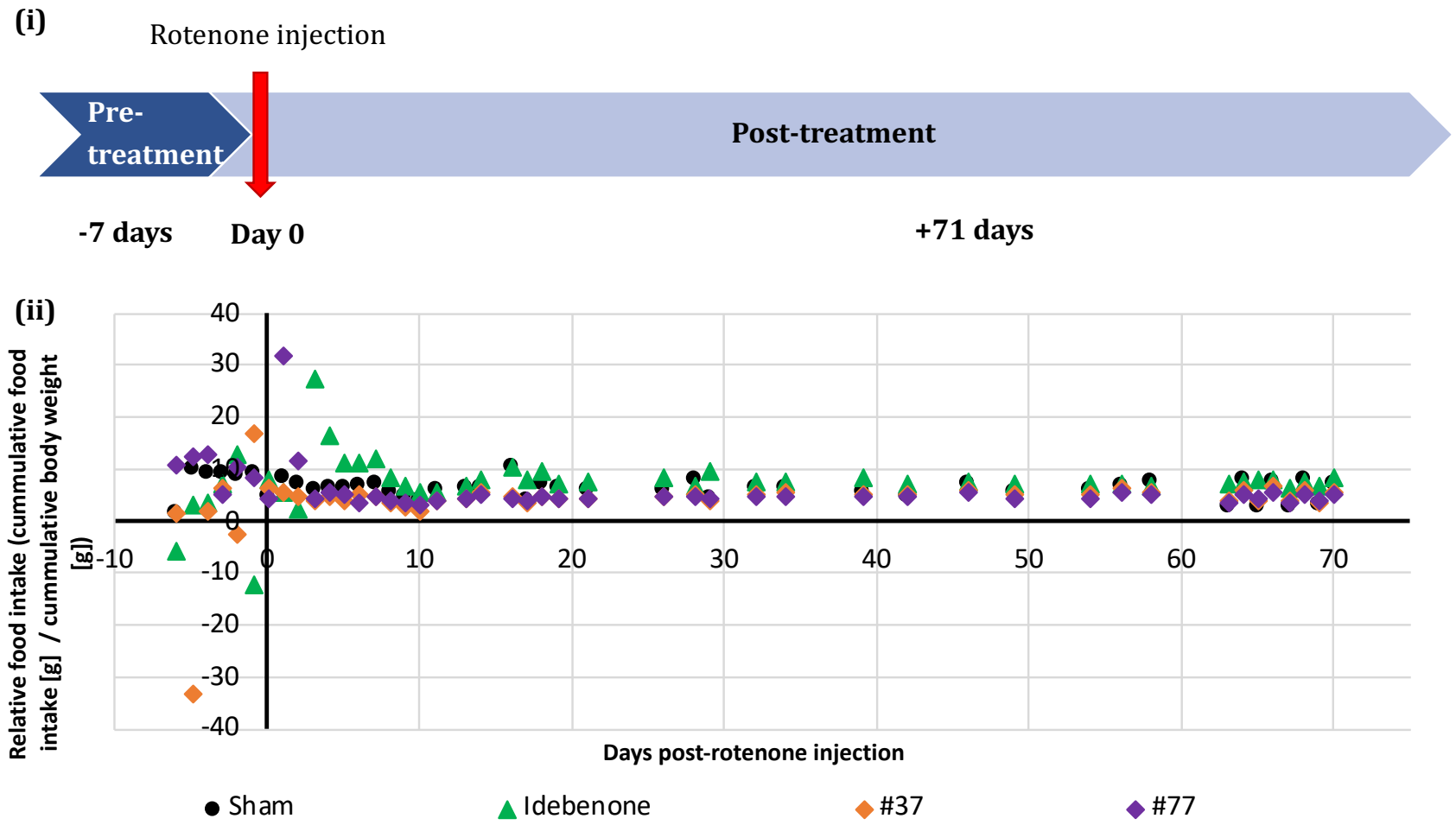


Figure 43: Influence of short-chain quinones (SCQs) on relative food intake of mice. (i) All mice were pre-treated for 7 d with test compound, prior to intraocular rotenone injection on day 0, followed 71 d post-treatment. **(ii)** Relative food intake of mice was measured as the ratio of cumulative increase in food pellet intake (g) to the cumulative increase in body weight (g). Each data point represents the average of 5-11 mice per treatment group (n=5-11).

3.4.4 The effect of SCQ treatment on rotenone-induced retinal damage

After the end of treatment period (71 d), mice were sacrificed and eyes were collected for histological analysis. In order to investigate the efficacy of the novel SCQs against the consequences of rotenone-induced toxicity, the number of RGCs and retinal thickness were quantified on H & E stained eye sections using ImageJ software.

The right, non-injected eye of the sham-treated mouse represented the molecular pathology of a normal, healthy mouse and was therefore used as internal control (Figure 44). The retinal layers are composed of the innermost GCL that hosts the RGCs. This is followed by the inner nuclear layer (INL) and outer nuclear layer (ONL) that are separated by the inner plexiform layer (IPL) and outer plexiform layer (OPL) respectively. The nuclear layers are attached to the photoreceptor (PR) layer that is composed of inner segment (IS) and outer segment (OS) (Figure 44).

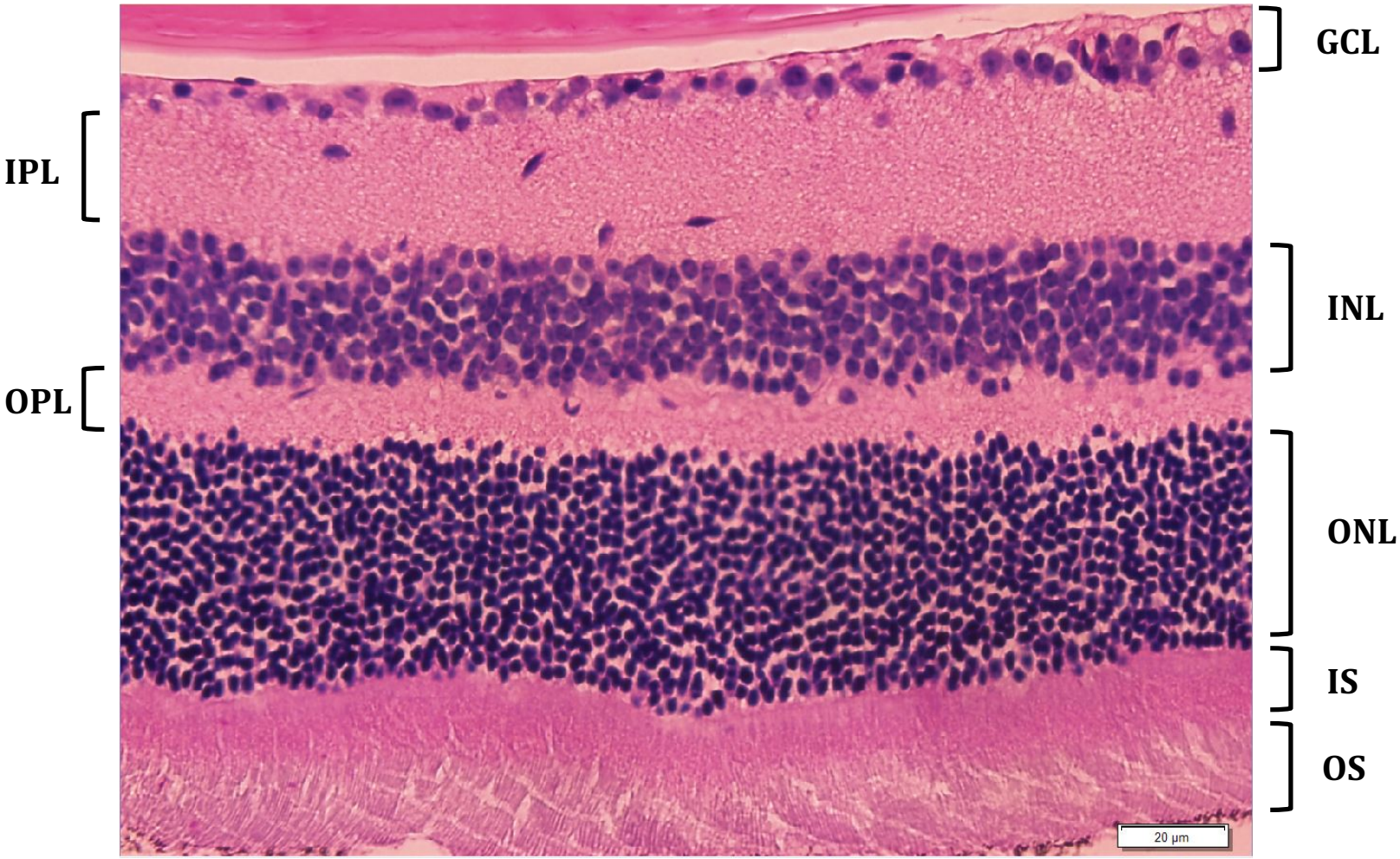


Figure 44: Retinal layers in mouse eye. The retina of a normal, healthy mouse is composed of the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and the photoreceptor (PR) layer that consists of the inner segment (IS) and outer segment (OS). Image was taken at 40 X magnification. Scale bar represents 20 µm.

3.4.4.1 The effect of novel SCQ treatment on rotenone-induced decrease in retinal thickness

In sham-treated mice, significant changes in retinal histology were evident from the images of H & E stained sections (Figure 45, a(i) versus a(ii)). Rotenone injection significantly reduced the retina thickness in left eye (Figure 45, a(i) versus a(ii)) when compared to right eye of sham-treated mice (54.11 ± 24.52 μm vs 118.99 ± 15.83 μm, Figure 46 i). It is worth noting that no significant changes in the retinal thickness of the non-injected, right eye were observed across all treatment groups (Figure 46 ii).

Upon treatment with idebenone, no significant differences were observed in the retinal thickness of the left-eye of idebenone-treated mice when compared to sham-treated mice (59.10 ± 21.19 μm vs 54.11 ± 24.52 μm, Figure 45 b(i) and b(ii), Figure 46 i). In contrast, treatment with both novel SCQs, #37 and #77 significantly protected against loss of retinal thickness (85.61 ± 24.78 μm, 92.84 ± 18.76 μm respectively) when compared to the retinas of sham-treated mice (Figure 45 c and d, Figure 46 i). Overall, in all treatment groups, 71 d post-rotenone challenge, the average retinal thickness of the left eye was significantly lower when compared to the right eye. For example, 54.11 ± 24.52 μm versus 118.99 ± 15.83 μm in sham-treated mice) (Figure 46 iii).

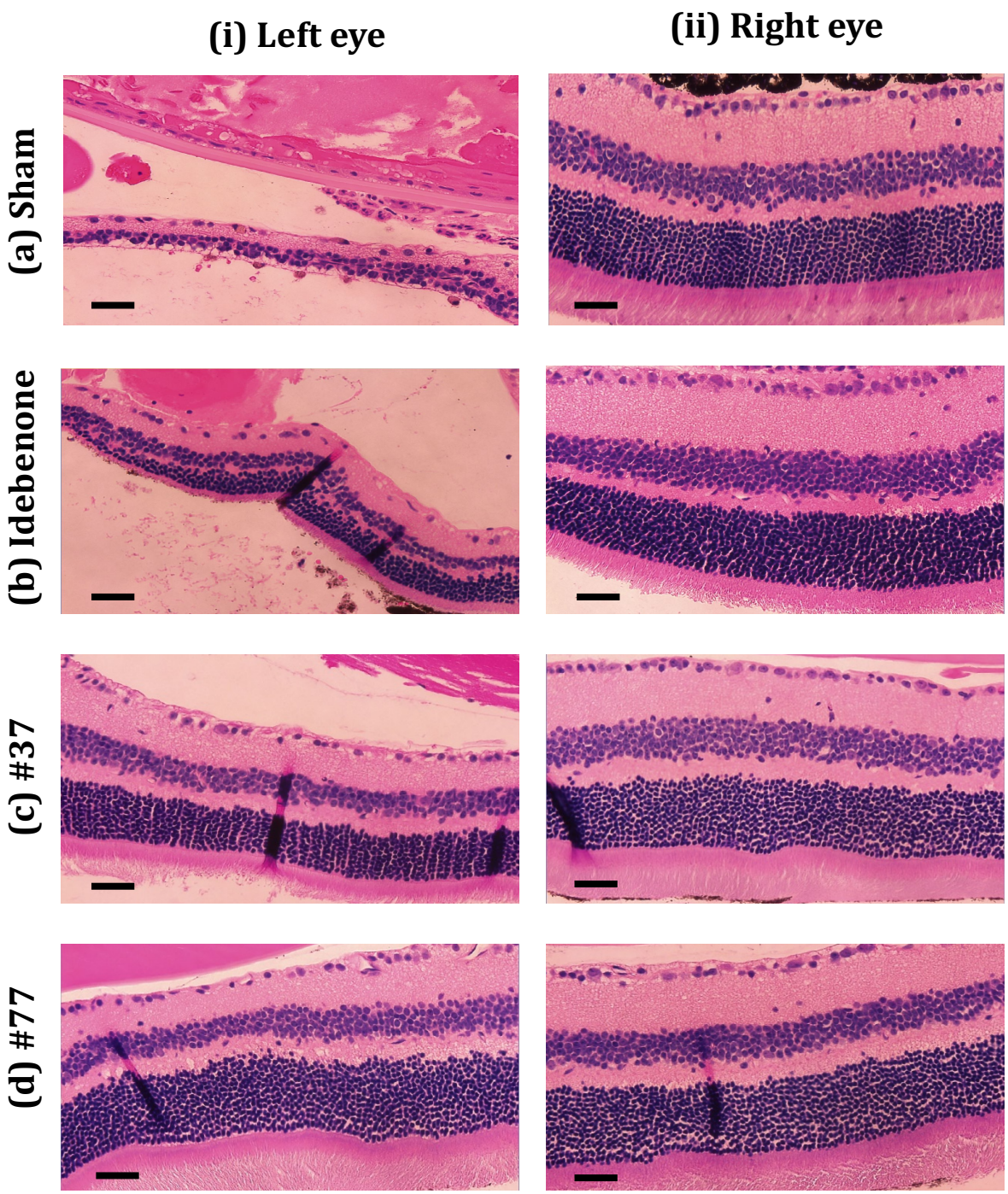


Figure 45: Histology of test-compound treated mice retina. Paraformaldehyde-fixed, paraffin-embedded, Haematoxylin (H) and Eosin (E) stained, 4 µm-thick retinal sections. The panel is composed of left (i) and right (ii) eye sections of one representative mouse from sham (a), idebenone (b), novel short-chain quinone (SCQ) #37 (c) and SCQ #77 (d) treated mice. All images were taken at 40 X magnification. Scale bar represents 20 µm.

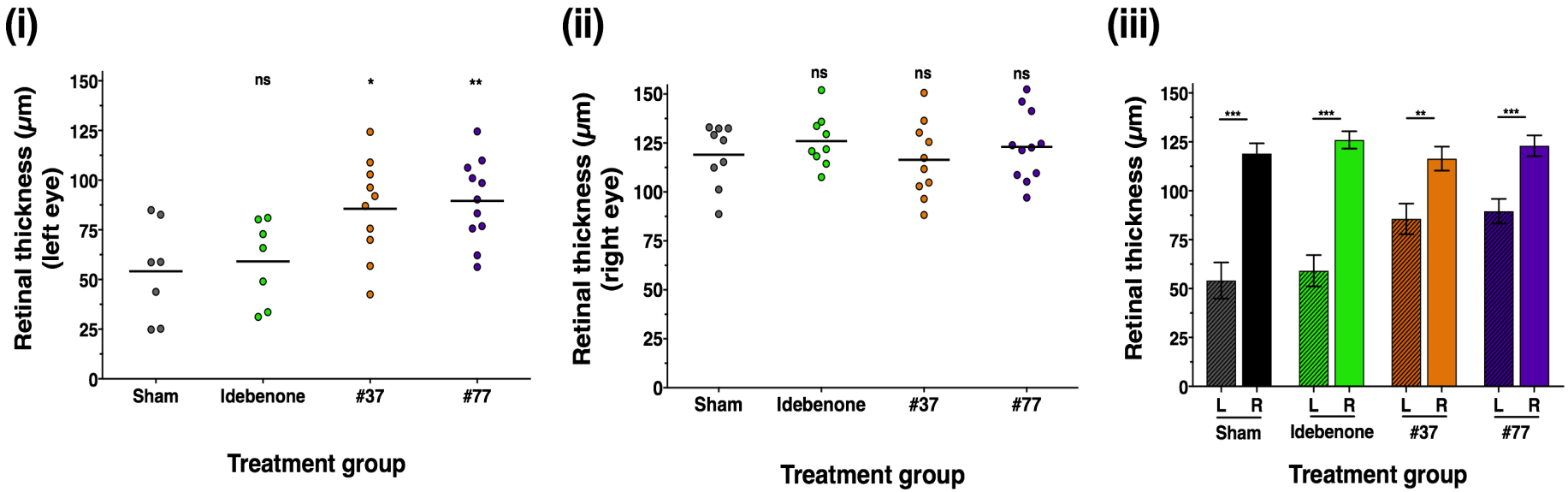


Figure 46: Quantification of retinal thickness of experimental mice. Average retinal thickness (μm) of left (i) and right eye (ii) measured from haematoxylin and eosin (H & E) stained, 40 X magnified image of 4 μm thick sections using ImageJ software. Data is presented as the mean within each treatment group (n=7-11 per treatment group). Each data point represents one mouse. Significance was calculated by comparing the average of each treatment group with sham-treated mice using Student *t*-test on GraphPad Prism software (* $p<0.05$, ** $p<0.01$). (iii) Comparison of retinal thickness of left versus right eye amongst different treatment groups. Data is presented as the mean ± SD within each treatment group (n=7-11 per treatment group). Significance was calculated by comparing the retinal thickness of left versus right eye within each treatment group using Student *t*-test on GraphPad Prism software (*** $p<0.005$).

L, left eye; R, right eye.

3.4.4.2 The effect of novel SCQ treatment on rotenone-induced loss of RGCs

In sham-treated mice, significant changes in the number of RGCs were evident from the images of H & E stained sections (Figure 45, a(i) versus a(ii)). Rotenone injection significantly reduced the number of RGCs/ mm in left eye (Figure 45, a(i) versus a(ii)) when compared to right eye of sham-treated mice (70.62 ± 19.92 vs 176.71 ± 56.89, Figure 47 i, ii). It is worth noting that no significant changes in the retinal thickness of the non-injected, right eye were observed across all treatment groups (Figure 47 i, ii).

Overall, in sham, idebenone and #77-treated groups, 71 d post-rotenone challenge, the average number of RGC/mm in the left eye was significantly lower when compared to the right eye. For example, 70.62 ± 19.92 RGC/mm in the left-eye versus 176.71 ± 56.88 RGC/mm in the right eye sham-treated mice (Figure 47 iii). Upon treatment with idebenone, no significant differences were observed in the number of RGC/mm in the left-eye of idebenone-treated mice when compared to sham-treated mice (75.74 ± 21.15/mm vs 70.62 ± 19.92/mm, Figure 47 i). Interestingly, treatment with both novel SCQs, #37 and #77 significantly protected against loss of RGC/mm (118.74 ± 55.34/mm, 106.55 ± 26.45/mm respectively) when compared to sham-treated mice (Figure 47 i). Interestingly, in the #37 treated group, despite the rotenone injection, no significant changes were observed in the number of RGC/mm when comparing the left with the right eye, (118.74 ± 55.34/mm versus 167.77 ± 51.60/mm, Figure 47 iii).

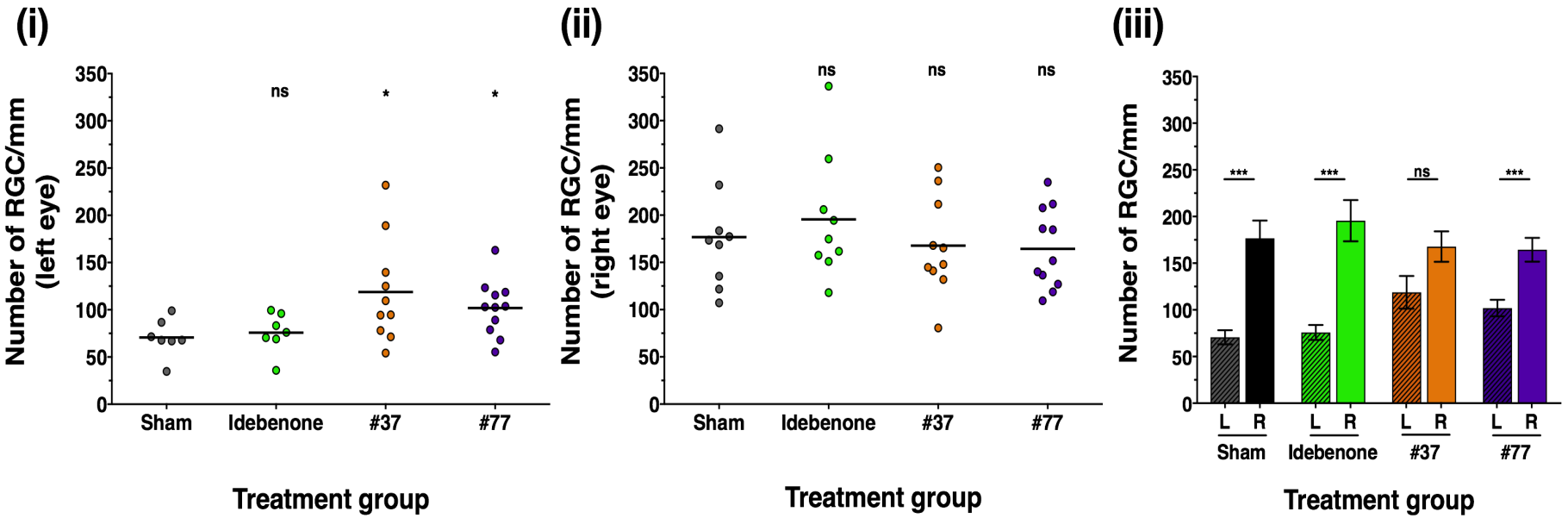


Figure 47: Quantification of retinal ganglion cells (RGC). Average number of RGC/mm of left (i) and right eye (ii) quantified from haematoxylin and eosin (H & E) stained sections at 40 X magnification. Data is presented as mean within each treatment group (n=7-11 per treatment group), each data point represents one mouse. Significance was calculated by comparing the average of each treatment group with sham-treated mice using Student *t*-test on GraphPad Prism software (* $p<0.05$, ** $p<0.01$). (iii) Comparison of number of RGC/mm in left versus right eye amongst different treatment groups. Data is presented as mean ± SD within each treatment group (n=7-11 per treatment group). Significance was calculated by comparing the number of RGC/mm in the left versus the right eye within each treatment group using Student *t*-test on GraphPad Prism software (*** $p<0.005$).

L, left eye; R, right eye.

3.4.5 Effect of novel SCQs on rotenone-induced loss of visual acuity.

Rotenone-induced reduction in retinal thickness and loss of RGCs could together result in the loss of visual acuity. Consistent with a previously published study (Heitz et al. 2012), intraocular injection of 5 mM rotenone into the left eye exclusively abolished the OMR of sham-treated mice within 1 d, when compared to the right eye (0.3 ± 0.7 versus 19.8 ± 4.7 head-turns, Figure 48). This result is indicative of lost visual acuity in the left (injected) eye. In contrast, no significant changes of visual acuity were noted in the right, non-injected control eyes, either before or after rotenone injection. Once the model of rotenone-induced vision loss was established in our laboratory environment, the same injection protocol was followed for all treatment groups to induce blindness. One day post-rotenone injection, no significant differences were observed in the OMR of the left eye of the SCQ-treated mice, when compared to sham-treated mice (Figure 48). Comparable to sham-treated mice, all SCQ-treated mice also showed a significant drop in visual acuity in the left eye, when compared to the right eye (Figure 48). On average, one day post-rotenone injection, only 0.4 ± 0.7, 1.4 ± 2.4 and 2.1 ± 3.2 head turns were counted in the left eye for idebenone-, #37- and #77-treated mice respectively, whereas the right eye showed 19.6 ± 5.8, 19.1 ± 3.3 and 18.3 ±6.7 head-turns respectively. Mice that did not show a significant drop in visual acuity upon rotenone injection, were excluded from the study (n=3, out of a total 43).

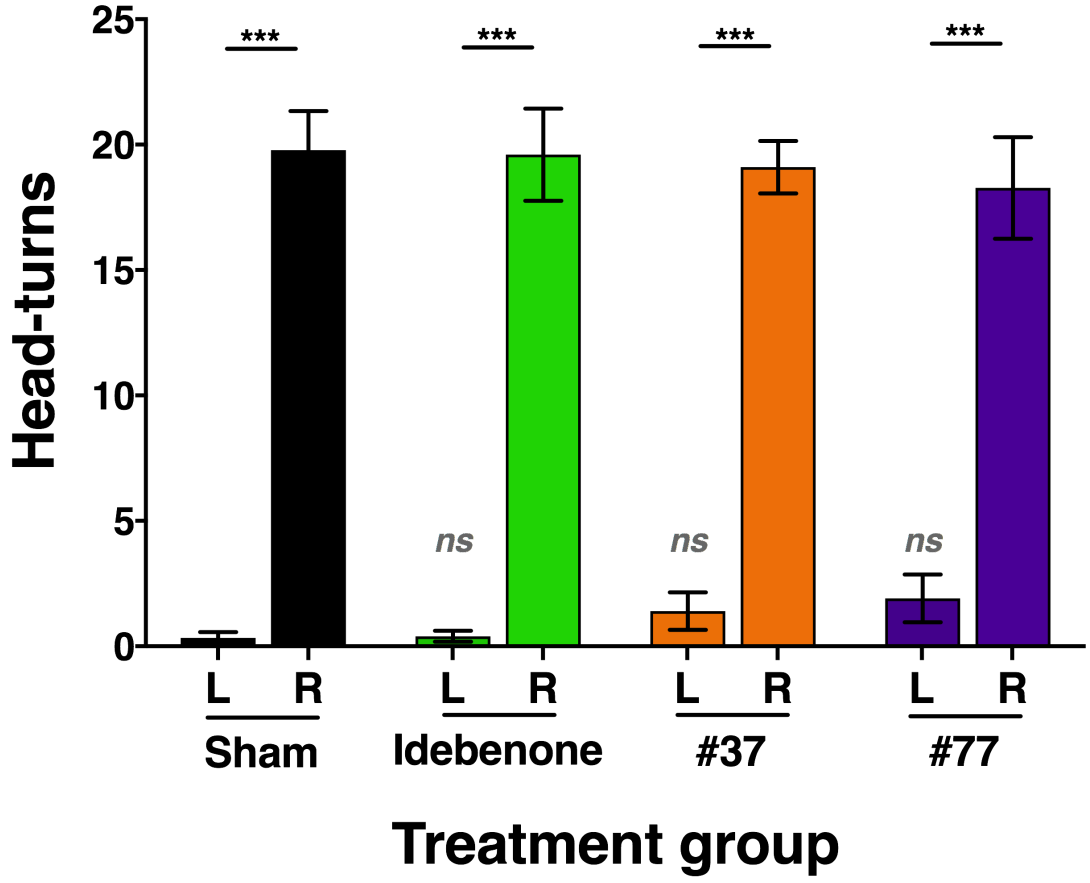


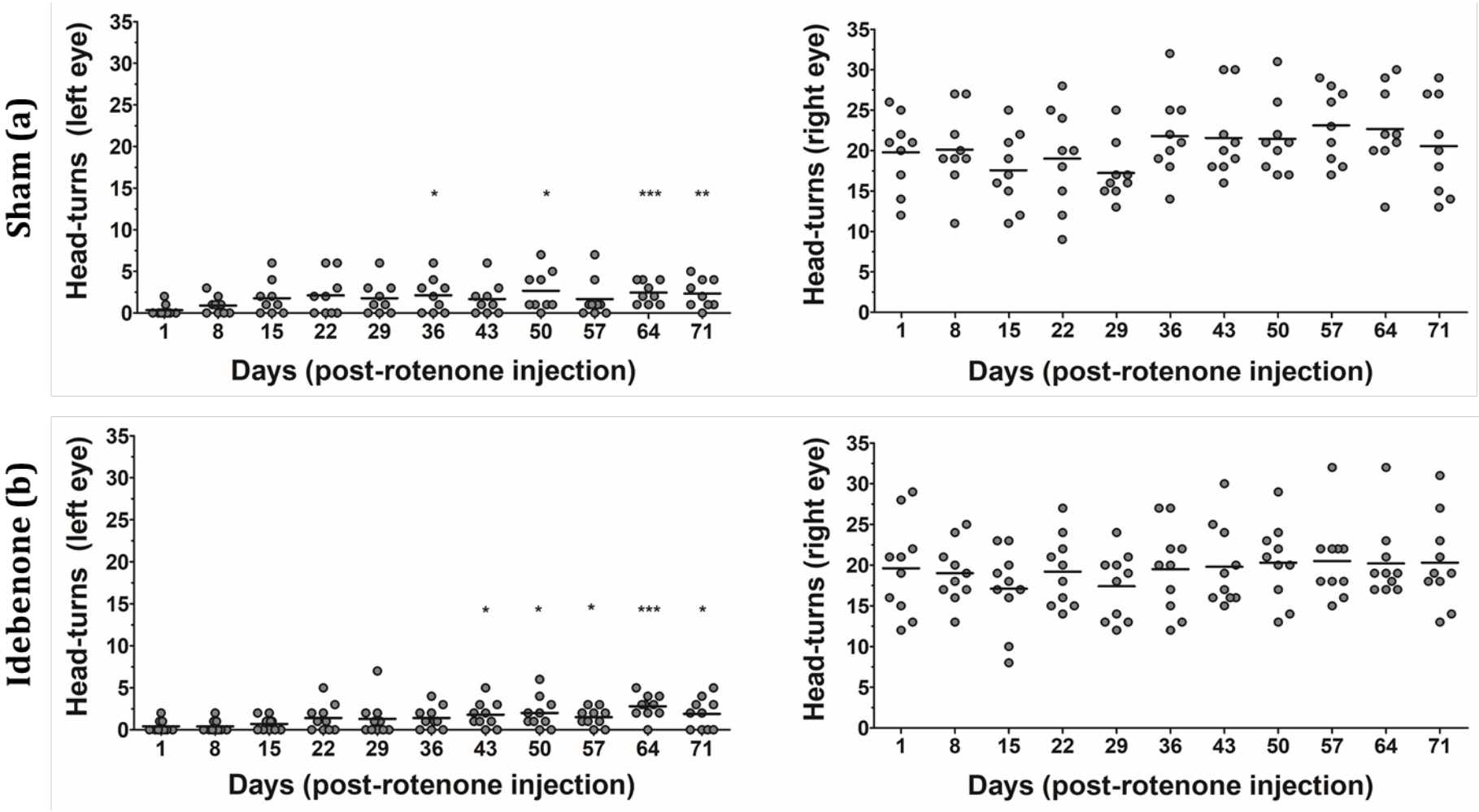
Figure 48: Effect of intraocular rotenone injection on visual acuity. Visual acuity of mice was measured one day after injecting 1 µl of 5 mM rotenone solution in the left-eye of all mice while the right eye remained as internal control for each mouse. Visual acuity was quantified by scoring the number of head-turns by mice while rotating the stripes of the optokinetic drum for 2 min in clockwise (left-eye, L) and anti-clockwise (right eye, R) direction. Each bar represents the head-turns corresponding to each eye of the mouse. Data is expressed as mean ± SD, n=9-11 mice within each treatment group. Significance was calculated by using Student *t*-test to compare number of head-turns of right versus left eye within each treatment group (black stars, p^{***} <0.001) and between left eye of sham versus left eye of SCQ-treated mice (grey, ns, non-significant).

Consistent with a significant reduction of retinal thickness and loss of RGCs in sham treated mice, a significant loss of visual acuity was observed (Figure 48). Replicating the natural phenomenon of LHON in clinical patients, some mice from the sham-treated group started to regain visual acuity spontaneously. When compared to one day post-

rotenone, significant improvements in visual acuity were seen after 5, 7, 9 and 10 weeks post-rotenone injection in sham-treated mice (Figure 49 a). No significant changes in visual acuity were observed in the non-injected, right eye of sham treated mice (Figure 49 a).

Although no significant changes were observed in retinal thickness or RGC numbers (Figure 46, 47) in idebenone-treated mice, significantly improved visual acuity was observed from six-weeks post-rotenone injection in the left eye when compared to 1 done day post-rotenone, with no significant changes observed in the right eye (Figure 49 b). On average, the visual acuity of idebenone-treated mice was less than 5 head turns in the left eye, compared to around 20 head turns counted in the right eye (Figure 49 b).

When compared to one day post-rotenone, mice treated with SCQs #37 and #77 on an average started to show significantly improved visual acuity from eight-weeks and two-weeks post-rotenone injection, respectively (Figure 49 c). Interestingly, only two mice from each #37 and #77 treated groups recovered visual acuity to the same levels as the non-injected, right eye of sham-treated mice (Figure 49 c, d), which is reminiscent of the patient-specific responses in idebenone-treated LHON patients. No significant changes in visual acuity were observed for the right eyes of mice from all treatment groups, when compared to sham-treated mice (Figure 49 a-d).


Sham (a)
Head-turns (left eye)
Head-turns (right eye)
Days (post-rotenone injection)
Idebenone (b)
Head-turns (left eye)
Head-turns (right eye)
Days (post-rotenone injection)

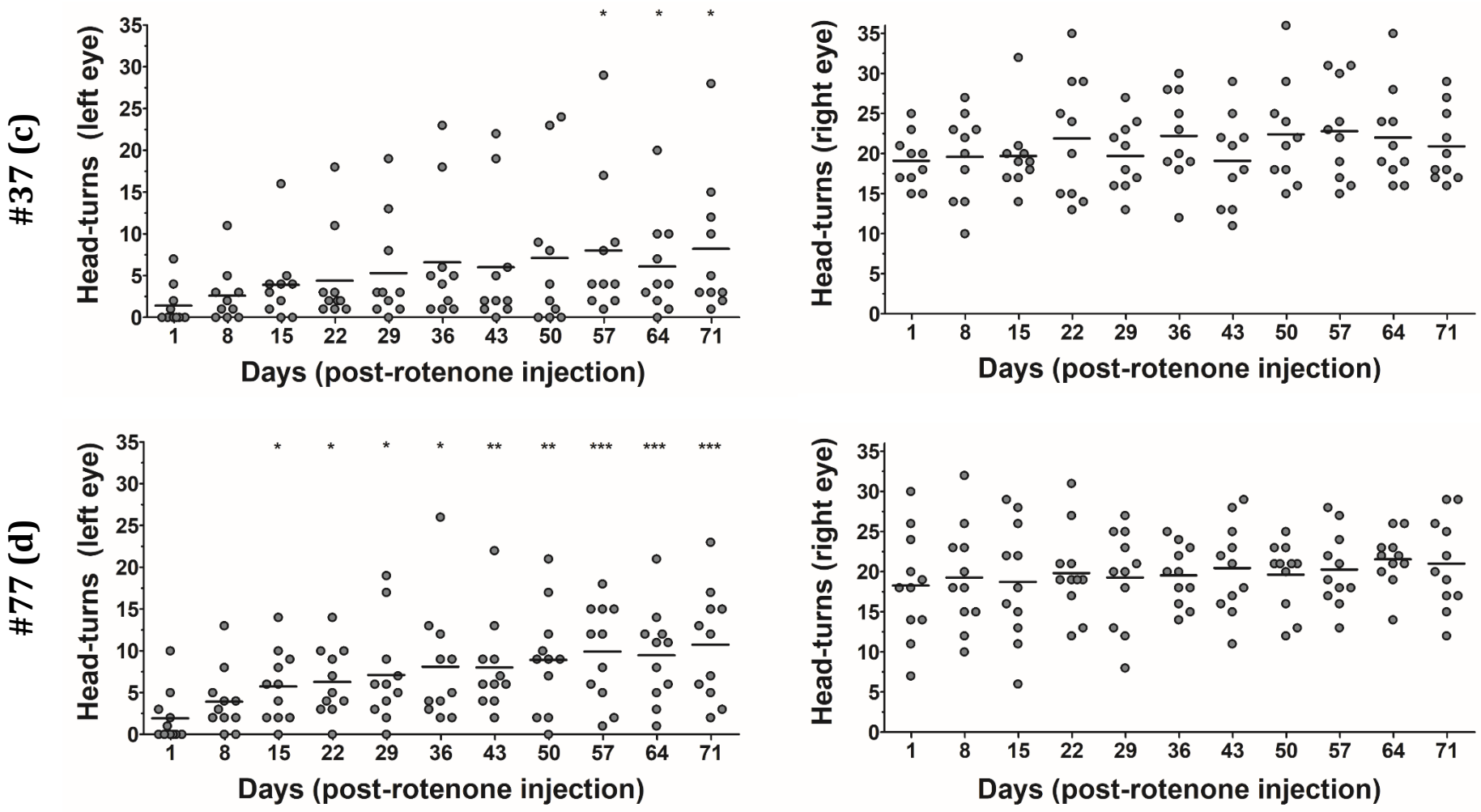

#37 (c)
Head-turns (left eye)
Head-turns (right eye)
#77 (d)
Head-turns (left eye)
Head-turns (right eye)
Days (post-rotenone injection)
1 8 15 22 29 36 43 50 57 64 71
0 5 10 15 20 25 30 35
* ** ***

Figure 49: Effect of SCQs on visual acuity following rotenone-induced vision loss. Following intraocular rotenone injection in the left eye, visual acuity was measured once every week over the entire study period for all treatment groups, i.e. sham (a), idebenone (b), #37 (c) and #77 (d). The right eye was used as internal control within each mouse. Visual acuity was quantified by scoring the number of head-turns while rotating the stripes of the optokinetic drum for 2 min in clockwise (represents the left-eye) and anti-clockwise (represents the right eye) direction. Each data point represents one mouse. Data is expressed as mean of n=9-11 mice. Significance was calculated using Student *t*-test to compare the number of head-turns against the number one day post-rotenone injection within each group (p^* <0.05, p^{**} <0.01, p^{***} <0.001).

Overall, calculating the average OMRs from the left eyes, no significant improvements in visual acuity was observed in idebenone-treated mice when compared to sham-treated mice, until the end of the study period (Figure 50). Importantly, when compared to sham-treated mice, significant improvements in visual acuity were observed in mice treated with #77 from only one-week post-rotenone injection, while #37-treated mice showed significant improvements five, eight and 10-weeks post-rotenone injection (Figure 50).

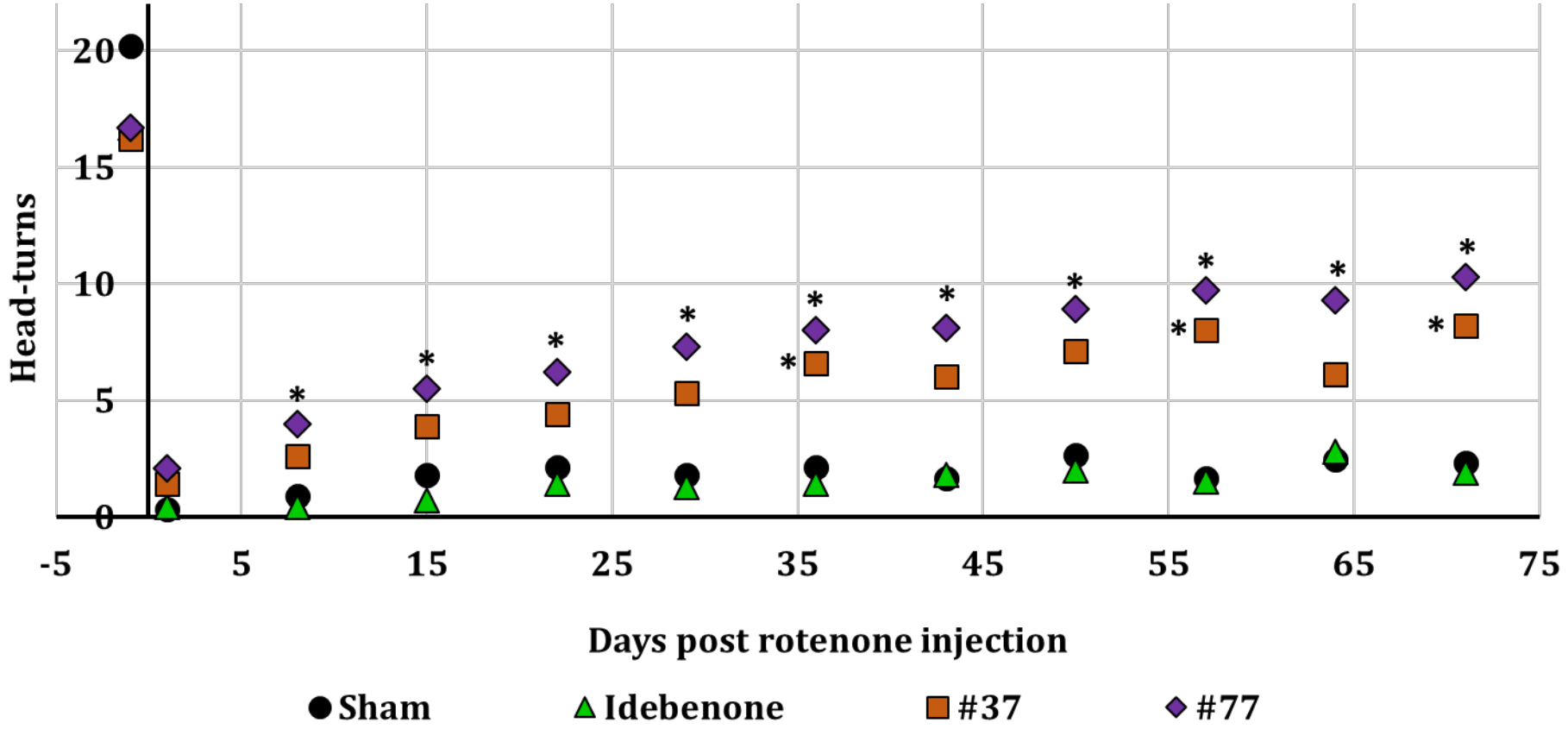


Figure 50: Influence of novel short-chain quinones (SCQ) on visual acuity after rotenone-induced vision loss. Visual acuity of mice was measured one day before and after rotenone injection into the left-eye of all mice. This was followed by visual acuity measurements once every week. The visual acuity of the left eye was quantified by scoring the number of head-turns while rotating the stripes of the optokinetic drum in clock-wise direction for in 2 min. Each data point represents one mouse. Data is expressed as the mean of n=9-11 mice. Significance was calculated using Student *t*-test to compare the number of head-turns against sham-treated mice at the particular time-point ($p^* < 0.05$)

3.4.6 Responder analysis for SCQ-dependent improvements of visual acuity after rotenone-induced vision loss.

One day after rotenone injection, the average number of head-turns in sham-treated mice was 0.7 ± 0.3. Therefore, mice who showed more than one head-turn 71 d post-rotenone injection were considered as responders. Consistent with the published literature (Heitz et al. 2012), spontaneous recovery of visual acuity was observed in sham-treated mice, with 65 % responders. However, as noted before these responders typically showed very low head-turn numbers overall. In contrast, the novel SCQs, #37 and #77 increased the percentage of responders to 90 % and 100 % respectively under conditions where idebenone treatment did not improve the percentage of responders (60 %) over sham-treated mice (Figure 51 i).

Due to spontaneous recovery of vision, mice from the sham-treated group showed a maximum of 7 head-turns during the entire treatment period (Figure 49 a). Therefore, I defined mice from the SCQ-treated groups that showed more than 7 head-turns as responders to SCQ-treatment, which were reported as "% responders to SCQ-treatment" (Figure 51 ii). When using this definition, 40 % and 70 % of mice responded to the novel SCQs #37 and #77 respectively, while no mice (0 %) responded to idebenone treatment (Figure 51 ii).

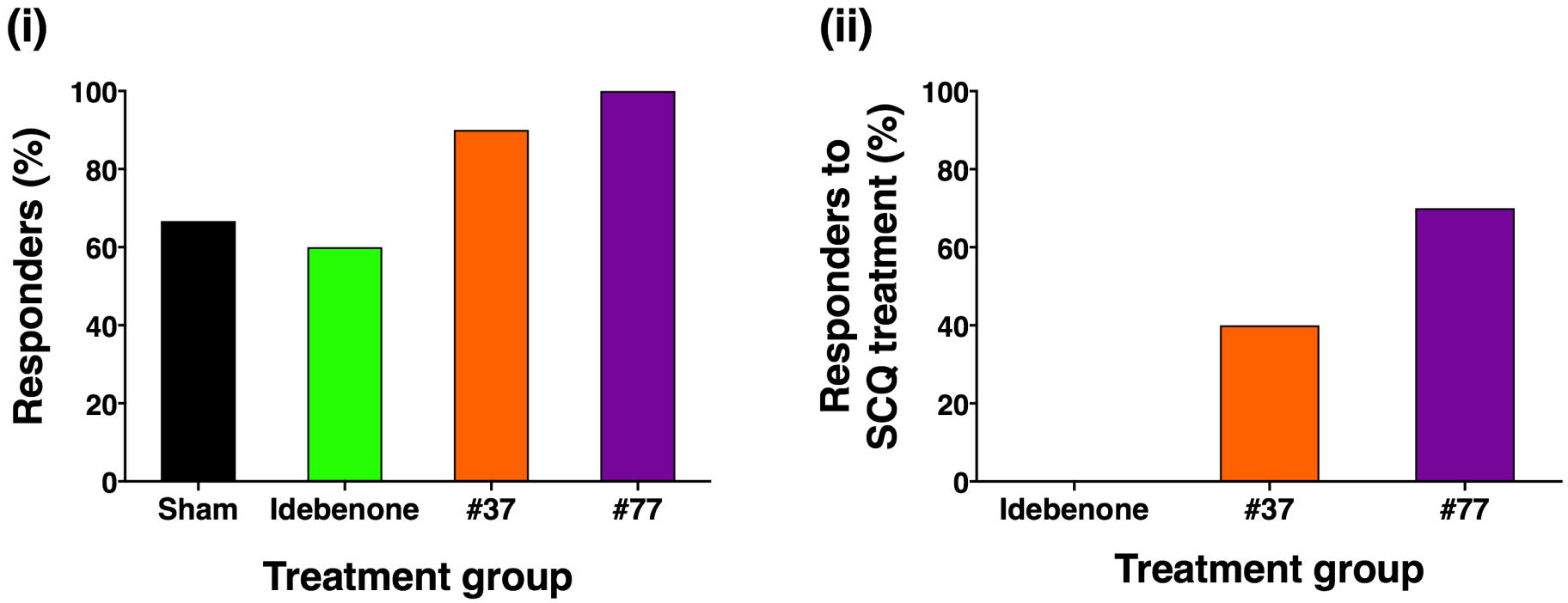


Figure 51: Responder analysis. At the end of the treatment period, 71 d post-rotenone injection, **(i)** mice showing more head-turns than sham-treated mice at 1 d post-rotenone (one head-turn) were considered as responders and were reported as Responders (%) within each treatment group and **(ii)** the number of SCQ-treated mice that showed more than the maximum number of head-turns showed by sham-treated mice throughout the treatment period (7 head-turns) were considered as responders to SCQ treatment and reported as Responders to SCQ treatment (%) (n=9-11 mice within each group).

3.5 Discussion

LHON is one of the most common mitochondrial disorders that is characterised by acute loss of visual acuity and loss of colour vision (Yu-Wai-Man et al. 2011). The disease progression of LHON can be divided into three stages (Figure 52). The first, pre-symptomatic phase of LHON is only characterised by peripapillary angiopathy without affecting visual acuity. The second, acute phase of the disease is initiated by one of the LHON mutations in combination with secondary genetic and environmental factors (Hudson et al. 2007, Kirkman et al. 2009). This phase is typically characterised by rapid loss of visual acuity, which is also associated with the swelling of retinal nerve fibre layer (RNFL), energy deficiency and oxidative stress. Increased oxidative stress due to accumulation of reactive oxygen species (ROS) and reduced oxidative phosphorylation (OXPHOS) and energy levels is believed to lead to RGC dysfunction (Chrysostomou et al. 2013, Gueven 2014, Mehta et al. 2013). This could lead to an inability by the RGC to transmit optical information to the brain despite the cells being still alive. Elevated ROS levels and resultant excitotoxicity over a prolonged period during the acute phase of the disease is believed to subsequently initiate the final phase of the disease progression that is characterised by the irreversible death of RGCs (Ghelli et al. 2003). The apoptotic loss of RGC occurs in the final, atrophic phase followed by the functional impairment of individual fibres, which leads to optic nerve atrophy (Barboni et al. 2010, Barcella et al. 2010). This is supported by histological post-mortem analysis of optic nerve samples from patients with LHON that show degeneration of retinal ganglion cells, demyelination and fibre loss in the optic nerve (Saadati et al. 1998). This hypothesis of discrete stages between onset of symptoms and irreversible vision loss helps to postulate a window of opportunity during which spontaneous vision recovery is (at least in theory) possible

(Gueven et al.2014). In some patients, spontaneous vision recovery can occur during the first year after disease onset (Acaroğlu et al. 2001, Spruijt et al. 2006, Stone et al. 1992). However, in some rare cases, spontaneous vision recovery was reported several years after disease onset (Spruijt et al. 2006, Stone et al. 1992). The potential window between disease onset and estimated spontaneous recovery is the area where potential therapeutics might be used to facilitate this naturally but rarely occurring process to restore visual acuity (Figure 52).

Based on this understanding of the LHON pathology, therapeutic options have been trialled in both preclinical, *in vivo* models of LHON and in clinical trials with LHON patients to actually restore or facilitate the naturally occurring mechanism of vision recovery. The SCQ idebenone was reported to protect visual acuity and retinal pathology of mice under conditions of impaired mitochondrial C-I by intraocular rotenone injection (Heitz et al. 2012). These protective effects of idebenone were partly replicated in a clinical trial in LHON patients. In this study, although not statistically significant, an increase in visual acuity was observed in some LHON patients (Klopstock et al. 2011), that was maintained even 30 months after idebenone treatment had been terminated. This protective effect of idebenone was mainly observed in patients where treatment was commenced less than one year after disease onset (Klopstock et al. 2013). These results could suggest that SCQ treatment might initiate the spontaneous recovery of visual acuity that is sustained for a long duration even after terminating the treatment. This hypothesis is supported by the results of the present study where our novel SCQs showed a significantly higher responder rates compared to the naturally occurring rate of vision recovery associated with this animal model.

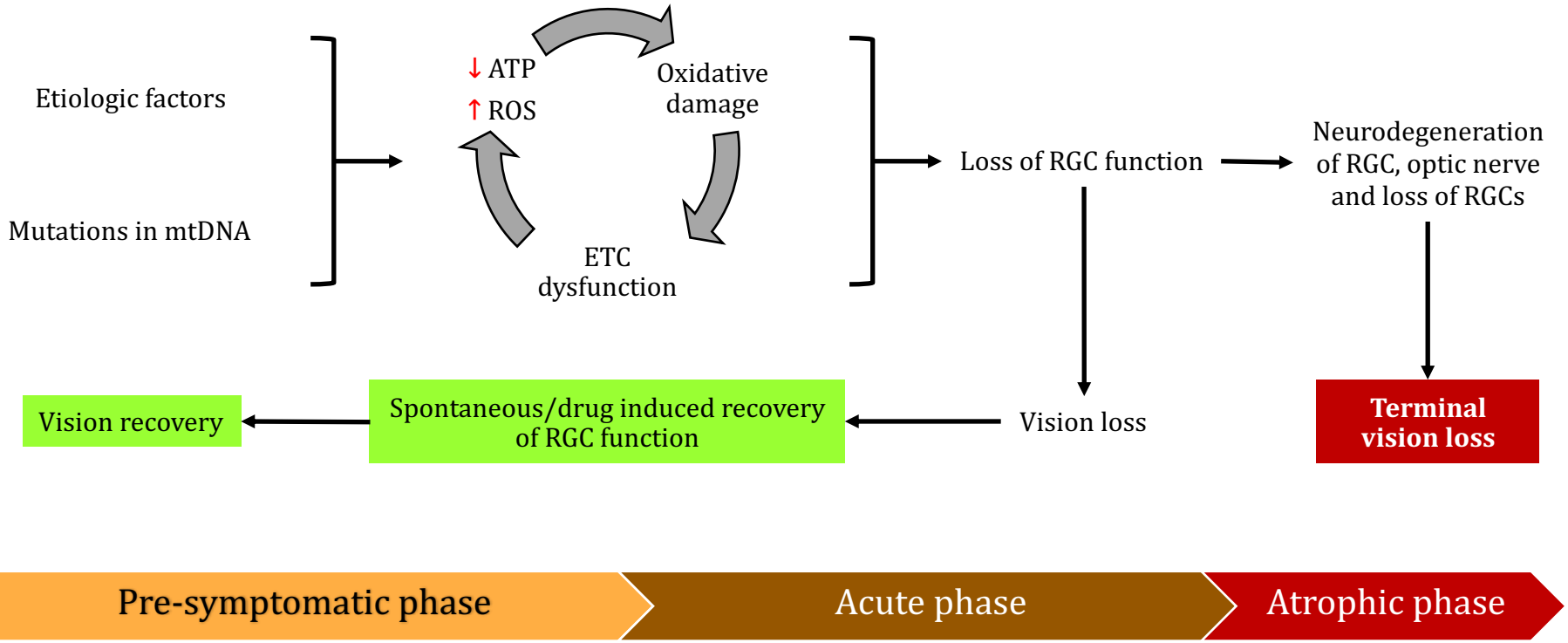


Figure 52: Simplified etiology of Leber's hereditary optic neuropathy (LHON). Primary mitochondrial DNA (mtDNA) mutations along with etiologic factors result in impaired energy (adenosine triphosphate, ATP) production, excess reactive oxygen species (ROS) and electron transport chain (ETC) dysfunction along with other risk factors to impair retinal ganglion cell (RGC) function in the acute phase of LHON. At this stage, vision may recover spontaneously or by therapeutic intervention. However, without interventions, RGC dysfunction eventually leads to RGC loss by an apoptotic cell death mechanism (Gueven 2014, Sanchez et al. 2016).

To provide supporting evidence for these protective effects of SCQs, it is essential to utilise an *in vivo* model of LHON that should allow us to investigate not only efficacy of therapeutic interventions, but also to generate detailed data on the timing of events and the molecular pathologies associated with different stages of disease.

Based on our current understanding of the LHON pathology, several murine models have been established that aim to mimic the characteristic pathologies of LHON. The approaches include mimicking mitochondrial dysfunction by genetic modelling or toxin exposure to inhibit mitochondrial C-I. Two genetic models of C-I dysfunction were developed by introducing mutations into two genes that encode subunits of C-I of the mitochondrial ETC, ND4 and ND6. However, these genetic models do not completely replicate the pathology of LHON and therefore lack face validity. In the mouse model characterised by a mutation in ND6 (P25L), synaptosome analysis revealed increased ROS production, while ATP levels in the synaptosomes remained unchanged (Lin et al. 2012). In another study conducted on rats harbouring a mutated ND4 gene, a degeneration of RGCs along with reduction of visual acuity were observed (Ellouze et al. 2008). However, it has to be noted that these genetic models are not necessarily replicating the situation in LHON patients, where mutation carrier does not necessarily develop the disease. For example, levels of penetrance as low as 26 % for males and 6 % for females have been reported for the m11778G>A mutation in Finland (Puomila et al. 2007). In addition, only 70 % of the RGCs in the ND4 model expressed the mutation for only up to 50 d, which severely limits the use of this model to test novel therapeutics over periods longer than 50 d (Ellouze et al. 2008).

Another approach to mimic LHON is the use of the C-I inhibitor, rotenone. Injection of rotenone-loaded microspheres into the optic layer of the superior colliculus of the brain

of rats, significantly reduced the visual acuity after 2 weeks, with an indication of elevated levels of ROS. However, this study was unable to measure any effects of this treatment on the number of RGC cells (Marella et al. 2010). Since this model is not only time consuming and requires the specialised production of microspheres (which necessitates control measures to avoid batch to batch variation and continuous stability controls), I used a simplified approach that included the direct intravitreal injection of rotenone. This approach was previously described to result in RGCs loss, reduced RNFL thickness, reactive gliosis and loss of visual acuity (Heitz et al. 2012, Rojas et al. 2008, Zhang et al. 2002). I also selected this model as it not only mimics the major pathological changes in LHON, but the rotenone concentration can be titrated to enable spontaneous vision recovery (unpublished data N. Gueven), similar to the situation in some LHON patients, which has not been described for the models listed above. In addition, this approach was previously used to demonstrate the protective activity of the benzoquinone, idebenone (Heitz et al. 2012). However, I have to acknowledge that this model, like the genetic models described above do not require additional (i.e. etiologic) factors that in conjunction with C-I inhibition leads to loss of visual acuity. In this sense, the results of this study cannot be directly translated to the situation in LHON patients.

So far, the efficacy of naphthoquinones to treat mitochondrial disorders has been discussed *in vitro* and in animal models of neurological disorders like epilepsy, since decreased ATP levels and elevated ROS levels due to mitochondrial dysfunction can increase neuronal excitability and induce seizures (Josey et al. 2013, Rahn et al. 2014). In these studies, vitamin K analogues reportedly reduced seizures with no observable toxicity in a mouse model of pentylenetetrazole (PTZ)-induced seizures. Since these compounds increased total cellular ATP levels in a mouse hippocampal cell line, the authors suggested that this activity is the potential mechanism of action of the

naphthoquinones of this study (Rahn et al. 2014). Although, these results support the use of naphthoquinones in treating mitochondrial disorders in general, the detailed mechanism of action of these compounds is yet to be understood.

In the current study, for the first time, I investigated the efficacy of novel naphthoquinones in a mouse model of rotenone-induced loss of visual acuity. A previous report used this model to show the protective activity of high doses of idebenone (2000 mg/kg) and included a 3-week pre-treatment period with idebenone prior to the rotenone challenge (Heitz et al. 2012). This pre-treatment with idebenone protected against loss of RGC, retinal thickness seven days post rotenone challenge and promoted a significant improvement of visual acuity seventy days post rotenone challenge (Heitz et al. 2012). In this study, a 3-week pre-treatment was selected, since at the time, no information was available on the pharmacokinetics of idebenone with regards to eye fluids and it was speculated that prolonged pre-treatment would provide stable trough levels (something that the same authors could disprove in the same study; N. Gueven unpublished observation; Heitz et al. 2012). Based on a better understanding of idebenone eye pharmacokinetics, but no information on the pharmacokinetics of our novel SCQs, we decided to pre-treat the mice for only one-week prior to the rotenone challenge. Similar to the previous study (Heitz et al. 2012), all SCQs were orally administered through the food. However, in contrast to the previous study (Heitz et al. 2012) I used only $1/10^{th}$ of the SCQ dose based on the significantly increased activity of our novel SCQs *in vitro*. In line with this reduction, all new SCQs were administered at a dose equivalent to 200 mg idebenone/kg, in contrast to the previously used 2000 mg/kg (Heitz et al. 2012).

In principle any SCQ pre-treatment could be seen to protect cells against a rotenone challenge. If this were the case, any subsequent changes in visual acuity could be the result of this difference in viability or cellular stress status one day after rotenone injection. This would also mean that instead of interpreting our data as a time-dependent recovery of vision by the novel SCQs, the results could simply be seen as a differential level of damage on day one with consequently different kinetics of recovery over the course of the study. However, no significant differences in visual acuity were observed in the left, rotenone-injected eye, one day after the rotenone challenge in SCQ treated mice, when compared to sham-treated mice. Although, it appears that the protective effects of SCQs are associated with their ongoing presence in the animals, the former possibility should not be ignored and assessed by future experiments that should test different pre- and post-treatment times. These experiments, as well as different doses were not included in the present study due to time constraints. Overall, the necessity of the one-week SCQ pre-treatment is unclear at this stage.

The potential benefits due to SCQ pre-treatment is in agreement with previously published literature where one day pre-treatment with idebenone reduced ROS levels and prevented apoptosis in cultured retinal neurons (Gil et al. 2003). In other study conducted on fibroblast cell lines from LHON patients, one day pre-treatment with idebenone significantly increased the activity of mitochondrial C-I (Angebault et al. 2011). Idebenone was also administered 3 weeks prior to injecting rotenone in an *in vivo* mouse model of LHON, in which significant improvement of retinal thickness and the number of RGCs, was observed 7 d post rotenone challenge (Heitz et al. 2012). However, in all these studies, the reason for pre-treatment was not discussed. In a recently published study, idebenone strongly induced the expression of Lin28A and significantly restored RGC survival and retinal thickness in combination with rasagiline (Lei et al. 2018). Lin 28A is

a highly conserved regulator of many cellular RNAs. With this function, Lin28A fundamentally affects metabolism, ageing, stress response, survival and the capacity for tissue repair (Shyh-Chang et al. 2013). Tissue repair capacity in particular is dependent on increased mitochondrial function (OXPHOS), improved glucose metabolism, increased insulin sensitivity, Akt-activation and reduced autophagy (Zhu et al. 2011), which could well explain the multiple SCQ-induced protective activities we and others observed in different pre-clinical models of mitochondrial dysfunction. However, Lin28A is mostly known for its binding to the pre-let-7 miRNA, which prevents its processing (Heo et al. 2008). Lin28A therefore reduces let-7 miRNA levels, which upregulates several oncogenes such as MYC, RAS and HMGA2 (Balzeau et al. 2017). Consistent with this function, ectopic Lin28 expression was reported to drive or accelerate tumorigenesis via a let-7 dependent mechanism (Heo et al. 2008) The idebenone-Lin28A connection is surprising and in stark contrast to the clinical data obtained with idebenone. Idebenone is characterized by an exceptionally good safety profile and after 1000s of patient-years no connection to any type of malignancy has ever been observed or reported. Nevertheless, in light of this recent report, it is essential to evaluate the potential cancer risk for our novel SCQs that could arise from a Lin28A-let-7-dependent mechanism.

Overall, it could nevertheless be hypothesised that SCQ-pre-treatment is needed to allow for a time-consuming gene expression step before SCQ-dependent protection is seen. However, this hypothesis has to be treated with extreme caution since the published evidence supporting the idebenone-induced Lin28A overexpression is so far based on a single report and does not fit the clinical evidence.

In this study, two selected novel, highly protective SCQs (Figure 41) were tested in a mouse model of rotenone-induced blindness. Although this model is widely used to

replicate many aspects of the LHON pathology, certain limitations must be acknowledged. Rotenone is believed to bind on the quinone-binding site of mitochondrial C-I, however, the exact binding site within C-I is not established yet (Friedrich et al. 1994, Prieur et al. 2001). Therefore, it is unclear if the sub-unit of C-I, inhibited by rotenone is same as the one encoded by genes that associated with LHON. This is of specific importance since not all mutations in C-I are associated with LHON but can be responsible for disorders with pathologies distinctly different to LHON. Although rotenone inhibits mitochondrial C-I and elevates ROS levels (Beretta et al. 2006, Li et al. 2003), rotenone also has off-target effects like any other small molecule, such as disrupting Ca^{2+} homeostasis (Freestone et al. 2009, Wang and Xu 2005) and induce apoptosis (Beretta et al. 2006, Li et al. 2003) that could conceivably modify the pathology of this animal model. It has to be noted that at present there is no perfect pre-clinical model for LHON that would replicate all patient characteristics such as incomplete penetrance, gender bias, need for additional factors in addition to the reproduction of the molecular pathology. Since the rotenone-injection model mimics the major pathological features of LHON, like loss of RGCs, reduced retinal thickness and loss of visual acuity and was used previously to test the efficacy idebenone (Heitz et al. 2012), this model was opted in the current study. It has to be acknowledged that a possible limitation of this animal model is the procedure of intraocular injection of rotenone. Since the injection was performed manually, unintentional errors could be introduced, such as leakage of rotenone out of the vitreous after injection, minor changes in applied rotenone dose (given that only one microliter is injected) or inaccurate angle of injection resulting in altered site of rotenone delivery. These factors can potentially lead to inaccurate rotenone dosing. This was the reason why mice without significant loss of visual acuity one day post-rotenone challenge were excluded from the study.

Nevertheless, I cannot exclude that intra-animal variation was the result of minor inaccuracies of the injection process.

At the end of the study period, while collecting eyes, processing tissues or cutting sections for histological analysis, unintentional structural damage to the retina was observed for some eyes. Sections, where obvious handling artefacts resulted in significant variability of the tissue histology, were excluded from the analysis since retinal detachment restricted the ability to identify and quantify the same area on all retinal sections. Initial H & E stained images showed obvious changes in the tissue histology between the rotenone challenged left versus non-injected right eye (Figure 45). Consistent with the previously published studies (Heitz et al. 2012, Rojas et al. 2008, Zhang et al. 2002), a significant loss of retinal thickness and RGC numbers was observed in sham-treated mice upon rotenone injection (Figure 46, 47). It is important to point out that due to the timing of sample preparation only seventy days after the rotenone challenge only a disease pathology reminiscent of the atrophic phase of the disease was observed in the animals that included loss of RGCs and reduced retinal thickness (Barboni et al. 2010, Barcella et al. 2010, Ghelli et al. 2003). In contrast, the acute phase of LHON would have been associated with elevated levels of ROS and swollen RNFL (Chrysostomou et al. 2013, Gueven 2014, Mehta et al. 2013) but was not assessed in this study. Nevertheless, despite showing signs indicative of the atrophic phase at the end of the study, spontaneous vision recovery was observed in sham-treated mice. This indicates that this model might be associated at an earlier time point with an acute phase similar to LHON patients, which suggests that recovery of visual acuity is principally possible. This also supports the idea that in this window of opportunity a pharmacological intervention to induce this physiological process is a possibility. Interestingly, both novel SCQs, #37 and #77 significantly increased visual acuity, retinal thickness and RGC numbers when compared

to sham-treated cells under conditions where idebenone did not show any significant changes. Unlike the previously published study (Heitz et al. 2012), the lack of protection by idebenone in our study is most likely the consequence of the significantly lower dose used.

Both loss of RGCs and reduced retinal thickness due to mitochondrial dysfunction results in the loss of visual acuity. This was demonstrated in our study where all mice drastically lost visual acuity one day after the rotenone challenge (Figure 48).

Unfortunately, neither my *in vitro* nor my *in vivo* data have shed light on the molecular mode of action of the novel SCQs. What was observed is that all the previously proposed modes of action such as antioxidant activity and effects on ATP levels are likely unrelated to the cytoprotective function of this class of compounds. Therefore, at this stage it is unclear if the novel SCQs are more potent than idebenone or act via a completely different mechanism. While Lin28A may be involved in the mechanism of action of novel SCQs, it raises the question about the necessity of a pre-treatment period with SCQs, which requires further detailed investigations and are currently underway in my supervisor's laboratory.

The fact that none of the SCQs induced any significant changes in the non-injected right eye suggests that the SCQs do not show any adverse effects *per se* in the absence of mitochondrial dysfunction. However, this result is in contrast to a previous study where visual function was impaired in wild-type mice treated with 2000 mg/kg idebenone compared to *Opa1* mutant mice (Smith et al. 2016). Although this effect was not statistically significant, it is in contrast to my results, which could be attributed to the use of a much lower dose of idebenone in our study.

Overall, our novel SCQs did not impose any obviously visible adverse effects on mice at the dosage administered for the treatment duration. Nevertheless, since our novel SCQs are naphthoquinones and therefore Vitamin K analogues in the widest sense, they could have potentially affected blood coagulation as vitamin K is integral to the blood coagulation cascade. Therefore, another limitation of this study that has to be acknowledged was that the effects of the novel SCQs on blood coagulation or other organ functions were not assessed. Further studies analysing the safety, tolerability, pharmacodynamics and pharmacokinetics of the novel SCQs in much more detail are required and in fact underway by another PhD student (Zikai Feng). In summary, my data in this chapter presents compelling evidence that supports the development of our novel SCQs towards clinical use in mitochondrial indications such as LHON but also describes the necessity for a significant safety assessment of this class of molecules in general.

Chapter 4: Conclusion

4.1 Conclusion

Mitochondria are multifunctional organelles that mainly satisfy the majority of cellular energy demands (Campbell and Reece 2005, Wallace et al. 2010), while also representing a major source of reactive oxygen species (ROS) (Maurya et al. 2015, Zorov et al. 2014). In addition, mitochondria contribute to thermoregulation (Chouchani et al. 2016, Nedergaard et al. 2001), Ca^{2+} homeostasis (Giorgio et al. 2017, Ichas et al. 1994) and regulate cell death mechanisms (Vakifahmetoglu-Norberg et al. 2017, Wojtczak and Zabłocki 2008). Any physical or chemical insult or a genetic predisposition that leads to mitochondrial dysfunction can result in a wide range of pathologies and disorders (Carelli et al. 2002, Gueven 2014, Legault et al. 2015, Lin and Beal 2006, Sadeghian et al. 2016). It is therefore surprising that only a very limited number of drug candidates are in development that aim to protect mitochondrial function. The chemical class of quinones is known for their antioxidant activity and their ability to restore cellular ATP levels (Antonenko et al. 2008, Erb et al. 2012, Smith and Murphy 2010, Yu-Wai-Man et al. 2017). In addition, quinones can act as signaling molecules and enzymatic co-factors upon bioactivation by cellular reductases (Ayer et al. 2015, Dominy and Puigserver 2013, Marriage et al. 2003). The short-chain benzoquinone, idebenone (Figure 14 ii) was developed by Takeda in the 1980s as a Coenzyme Q_{10} (CoQ_{10}) analogue with the aim to protect energy generation and act as an antioxidant under conditions of mitochondrial dysfunction (Miyamoto et al. 1999, Suno and Nagaoka 1984). These two activities of idebenone were largely postulated based on the assumption that idebenone, as a CoQ_{10} analogue, would share the known biological activities of CoQ_{10}. Unfortunately, this assumption was never really questioned (Gueven et al. 2015). Although, idebenone did not produce consistent results in the early clinical trials in Alzheimer's disease patients

(Gutzmann and Hadler 1998, Thal et al. 2003), later trials in mitochondrial diseases were more encouraging (Klopstock et al. 2011, Liu and Wang 2014, Rudolph, Dimitriadis et al. 2013). A number of small trials with idebenone and other short-chain quinones (SCQs) overall support the hypothesis that targeting mitochondrial function could in principle be beneficial to treat diseases associated with mitochondrial dysfunction (Brzheskiy et al. 2015, Enns et al. 2012, Gueven et al. 2017, Sadun et al. 2012).

However, despite some encouraging protective effects by SCQs seen in pre-clinical and clinical trials, no causative modes of action have been scientifically confirmed for this class of molecules that go beyond pure associations. This lack of understanding is not only a major limitation for the rational development of improved SCQs but also limits our ability to predict their possible side effect profiles. Therefore, the real and urgent need to develop improved drug candidates tailored to patients with specific mitochondrial diseases is limited by the lack of fundamental understanding around the molecular mode(s) of action of SCQs.

In this study, we successfully developed a range of novel SCQs based on a naphthoquinone scaffold by an iterative medicinal chemistry drug discovery approach. These novel SCQs were highly effective in protecting cellular viability and normalising ATP levels in the presence of a mitochondrial C-I inhibitor, *in vitro.* At the same time, these compounds also influenced lactate and beta-hydroxy butyrate (BHB) levels in cell culture supernatant but did not affect basal levels of lipid peroxidation. Importantly, none of these measured parameters correlated with the cytoprotective activities observed. Therefore, in contrast to the current, possibly over-simplified assumption that rescuing ATP levels and reducing oxidative stress are central to restoring cell viability by SCQs, my results suggest that SCQs either act in a more complex fashion where different

compounds of the same chemical class could have one or several different mode(s) of action or could involve entirely new modes of action. These novel modes of action could involve two recently described signaling pathways that are influenced by SCQs, namely inhibition of p52Shc and activation of Lin 28A (Figure 37).

In the present study, I was able to generate a structure activity relationship (SAR) for the particular class of SCQs used in the present study, which was never reported in this amount of detail before. The precise combination of a naphthoquinone core with a methyl group and an alkyl side chain with specific functional groups of balanced charge and polarity were identified as the main determinants of their cytoprotective activity of this class of compounds.

To translate my *in vitro* results (Chapter 2) to an *in vivo* mitochondrial disease model, I investigated the efficacy of two selected novel, highly protective naphthoquinones (#37 and #77) in a mouse model of LHON (Chapter 3). Consistent with their cytoprotective activity *in vitro*, both novel SCQs, #37 and #77 significantly increased visual acuity, retinal thickness and protected retinal ganglion cell (RGC) numbers. Importantly, under these conditions idebenone did not show any significant protection. In fact, *in vivo* protection by our SCQs was observed at $1/10^{th}$ of the dose of idebenone used in previously published study (Heitz et al. 2012). This suggests that our novel SCQs are either significantly more effective than idebenone or act via a completely different mechanism. Comparable to the very good *in vivo* safety profile of idebenone, our novel SCQs did not result in any obviously adverse effects over the treatment duration.

While the exact molecular mode of action of the novel SCQs is still unclear, for the first time, my results strongly suggest that all the previously proposed modes of action such as antioxidant activity and effects on ATP levels are likely unrelated to the *in vitro*

cytoprotective function of this class of compounds. Nevertheless, the *in vitro* cytoprotective ability of our novel SCQs could be successfully translated to an *in vivo* model of mitochondrial dysfunction induced loss of visual acuity. Therefore, my data presents compelling evidence that supports the development of some selected SCQs towards clinical use in mitochondrial indications such as LHON. However, further characterisation of our SCQs with regards to general ADME characteristics and toxicity will be required to support this selection process.

4.2 Limitations of the Study

The current study aimed to develop and characterise novel SCQs for their ability to protect against mitochondrial dysfunction and work towards understanding their SAR and mode of action. Although I have addressed all the aims and objectives, a number of limitations inherent to this study need to be considered.

A common approach to drug discovery for mitochondrial disorders is the use of cybrid cell lines that harbour different mtDNA with the same nuclear DNA background. Therefore, trans-mitochondrial cybrids carrying one of the three LHON mutations that showed C-I dysfunction-induced oxidative stress (Carelli et al. 2004, Floreani et al. 2005, Wong et al. 2002) could have been used to characterise the protective activities of our novel SCQs. However, a consistent reduction of cellular ATP levels could not be demonstrated in LHON cybrids (Korsten et al. 2010). Furthermore, the tumor cells used to generate the available LHON cybrids are characterized by extremely low levels of NQO1, which would have significantly impaired the bioactivation of our compounds (Brown et al. 2000). Primary cell lines are also associated with other problems such as senescence, and unsteady growth rates, which can be avoided by using a cell line.

Moreover, the exact patho-mechanisms of diseases associated with mitochondrial dysfunction are not clearly known at present. A multitude of factors such as mutations and polymorphisms in nDNA and mtDNA, mitochondrial haplotype, epigenetic changes in tumor cells and environmental factors all affect disease symptoms and etiology (Burté et al. 2015, DiMauro et al. 2013, Larsson and Clayton 1995, Vafai and Mootha 2012, Zeviani and Di Donato 2004). These limitations make it difficult to generate a cell culture model of a specific mitochondrial disease that shows sufficient face-, construct- and

predictive validity (Gazdar et al. 2010). However, the availability of a reliable cell culture model was essential to consistently perform comparative studies over extended periods of time during the duration of my project. Therefore, I used a widely used hepatic cell line combined with a well-known mitochondrial toxin to generate a reliable model of mitochondrial dysfunction. Moreover, since quinones are known to undergo first-pass metabolism (Becker et al. 2010, Bodmer et al. 2009), I have chosen HepG2 cells to characterise our novel SCQs.

While cybrid cells largely do not express NQO1 at detectable levels, HepG2 cells on the other hand are known to express higher levels of NQO1 compared to normal liver cells. This overexpression could conceivably over-emphasize results with regards to SCQ bioactivation and metabolic activity (Cresteil and Jaiswal 1991). Therefore, my results cannot be easily extrapolated to other established cell lines containing low NQO1 levels such as neuronal cells (RGC5 or SHSY5Y) or human embryonic kidney cell line (HEK293). Despite the high NQO1 levels of HepG2 cells, the dependence of quinones on NQO1 for their cytoprotective ability has not been proven beyond doubt. The dependence of idebenone reduction by NQO1 for acute rescue of ATP levels in rat myoblasts has been published (Erb et al. 2012). However, the same research team showed cytoprotection of RGC5 cells (that only contain minimal levels of NQO1) by idebenone in the presence of rotenone (Heitz et al. 2012). This contradiction questions at least the dependence on NQO1 for the reduction of idebenone to enable its cytoprotective activity but does open the possibility that other reductases could compensate for a loss of NQO1. The possibility that the reduction of SCQs by cellular reductases is not necessarily required for their cytoprotective ability is supported by my results that failed to demonstrate a correlation between reduction and cytoprotection.

To measure the cytoprotective ability of the novel SCQs, I have used only a single mitochondrial C-I inhibitor, rotenone to induce a defined state of mitochondrial dysfunction, similar to previously published studies that tested idebenone and its analogues (Erb et al. 2012, Giorgio et al. 2012, Haefeli et al. 2011). Idebenone is believed to rescue ATP levels by bypassing mitochondrial C-I, in the presence of rotenone-induced mitochondrial dysfunction (Haefeli et al. 2011). While this is also true for other C-I inhibitors such as metformin (Haefeli et al. 2011, unpublished observation), idebenone is unable to protect ATP levels in the presence of other mitochondrial toxins such as antimycin or oligomycin that either inhibit C-III or block the ATP synthase respectively (Erb et al. 2012). Since our novel SCQs belong to the same chemical class as idebenone, we hypothesized that they would work by a similar mechanism. Moreover, most mitochondrial disorders and diseases with mitochondrial dysfunction are characterised by C-I dysfunction (Lin and Beal 2006). Therefore, I screened all novel SCQs against rotenone-induced mitochondrial dysfunction, which also allowed me to easily translate my *in vitro* results to an *in vivo* model of LHON that uses rotenone to induce vision loss in mice.

In this study, all novel SCQs were characterised *in vitro* at 10 μM in the initial screen. Bearing in mind the large number of compounds to be characterised, efficacy of the novel SCQs was measured at a single concentration, similar to previously published studies using idebenone (Erb et al. 2012, Haefeli et al. 2011). Based on my results, our compounds showed significantly better *in vitro* cytoprotection compared to idebenone and restored visual acuity of mice at $1/10^{th}$ the dose of previously published study using idebenone. This suggests that our novel SCQs may be more potent compared to idebenone. While some SCQs were tested at lower doses, the efficacy of other cytoprotective SCQs when used at lower concentrations is yet to be tested.

To measure the cytoprotective ability/toxicity of drugs the standard dye-based, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay frequently used. This assay assumes that mitochondrial dehydrogenases of viable cells reduce the MTT-tetrazolium salt to formazan, which is measured optically as a surrogate marker of the number of living cells (Chan et al. 2013, van Meerloo et al. 2011). However, compounds that increase cellular NADH levels, can enhance the production of formazan resulting in false negative results (Chan et al. 2013). MTT is also known to be reduced outside mitochondria in the extracellular environment, resulting false negative results (Bruggisser et al. 2002). The rate of reduction of MTT is also influenced by the concentrations of glucose, pyridine and even the pH of the culture medium (Vistica et al. 1991). In addition, the reduced form of quinones (hydroquinones) can also directly reduce the MTT dye and similar redox dyes such as WST-1 (N. Gueven, unpublished data; Tan and Berridge 2010) (Figure 16), which can result in the generation of false negative results (Petty et al. 1995). Since our novel SCQs are reduced by cellular reductases, influenced cellular metabolic pathways (Chapter 2) that could alter glucose concentration in the cell culture media, all of which are likely to alter formazan production, this experimental approach was not followed.

Another standard assay used to measure cell viability is by staining cells using dyes such as trypan blue. Unlike in healthy cells, damaged membranes would allow the bulky, charged dye molecules to enter within the cytoplasm and stain the cells purple-violet. This can be identified under a bright-field microscope (Cook and James, 1989). However, a serious limitation of this assay is that most cytotoxic agents initiate the damage intracellularly and membrane damage may not be evident immediately. Therefore, this assay might underestimate cytotoxicity (Hynes et al., 2003). Moreover, this assay relies on careful preparation of single cell suspension of cells and accurate counting of

individual samples using a hemocytometer. Consequently, this assay is not suited for higher throughput testing. In light of the large number of compounds that were assessed in parallel and the uncertainty of the accuracy of results associated with this assay, this experimental approach was avoided.

Instead, an ATP-based assay was chosen to measure cell viability. As ATP levels degrade rapidly during the process of cell death, ATP-dependent luminescence per well was measured as a readout of viability, which was then normalised to the luminescence generated by sham-treated cells for comparison. This assay was also selected since ATP-based assay showed higher sensitivity, linearity and reproducibility when compared to MTT assay in a previous study (Petty et al. 1995).

Although, ATP-measurement was employed for assessing cytoprotection and acute rescue of ATP levels, both assays are not assessing the same due to their inherent experimental designs. Cytoprotection was measured (after 2 days of preincubation with SCQs) one day after a seven-hour rotenone challenge, while in contrast acute rescue of ATP levels was measured after only one hour of co-exposure of cells to SCQs and rotenone. The independence of both assays is supported by the obvious lack of correlation between the results from these two assays (Figure 30). While some compounds combined good ATP rescue activity and poor cytoprotection, others, especially those with amino acid and alcohol side chain moieties, were highly cytoprotective and rescued ATP levels to more than 80 %. This led us to synthesize more compounds with these side-chain functionalities that subsequently populated the top right corner of the plot (Figure 30). Therefore, despite of a subjective correlation in this figure, the correlation plot of cytoprotection versus acute rescue of ATP levels for all SCQs (Figure 30), does not

demonstrate a real correlation between the two parameters but is a direct consequence of repeated rounds of compound optimization.

As a typical approach to measure the anti-oxidative capacity of novel SCQs in a cellular context, oxidative stress could be induced by pro-oxidants to overcome the in-built cellular antioxidant defense mechanism. However, as the main function of liver cells is to detoxify xenobiotics, these cells naturally display significantly higher defense mechanisms and tolerance to oxidative stress (Cresteil and Jaiswal 1991, Marroquin et al. 2007, Mersch-Sundermann et al. 2004). Consistent with this reasoning, no significant increases in oxidative stress-induced lipid peroxidation were observed upon treating HepG2 cells with H_2O_2 in this study (data not shown). Instead of inducing ROS by external addition of pro-oxidants, we measured the effect of SCQs on basal levels of lipid peroxidation. This indirect measure for antioxidant capacity was chosen over traditional assays that use redox dyes such as CM-H2DCFDA, since idebenone can interact with this redox dye to completely quench its fluorescence independent of a cellular context (N. Gueven unpublished observation, R. Haefeli, PhD thesis, unpublished). Since our novel SCQs belong to the same chemical class of quinones and could therefore have similar characteristics, I selected basal lipid peroxidation as a readout for antioxidant capacity as previously reported for myocytes (Erb et al. 2012). Since liver cells display significantly different defense mechanisms and tolerance to oxidative stress, these results may not be replicated by other cells. I can therefore not exclude the possibility that the novel SCQs may display potent antioxidant activity in other cell lines with lower tolerance to oxidative stress in contrast to HepG2 cells, where novel SCQs did not significantly influenced basal lipid peroxidation levels.

It has to be noted that HepG2 cells do not represent normal tissue responses as they originate from a hepatic cancer and have a very high glycolytic capacity (Marroquin et al. 2007). Increased glycolysis requires increased levels of NAD^+ that are needed for the central step of glycolysis, the conversion of glyeraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Typically, this NAD^+ is supplied by the conversion of pyruvate to lactate, which therefore leads to lactate accumulation (Figure 8 from Chapter 1) (Bodmer et al. 2008, Brewer 1998, Davidson et al. 2009, Dienel and Hertz 2001, Kaufmann et al. 2004, Rehncrona et al. 1981). Consequently, the non-significant changes to basal lactate levels that I found associated with some SCQs in cell culture supernatant might be impossible to extrapolate to other cell lines.

The current study represents the initial characterisation of a range of novel SCQs, as first step towards the identification of the best compounds to treat mitochondrial dysfunction. Given the large number of compounds to be characterised and a general difficulty to assess *in vitro* toxicity of quinones due to their interference with practically all standard viability assays, it was not possible to conduct a detailed investigation into the intrinsic toxicity for these compounds, which has to be regarded as another limitation of this study. Out of the best compounds, I selected two that showed sufficient differences in chemistry to confirm their protective activity *in vivo.* While systemic administration of these compounds did not show any overt signs of toxicity, another PhD student (Zikai Feng) will conduct a more detailed assessment of their *in vivo* toxicity.

To translate my *in vitro* findings to an *in vivo* model, two selected novel, highly protective SCQs were tested in a mouse model of rotenone-induced blindness. Although this model was used previously to replicate many aspects of the LHON pathology, certain limitations must be acknowledged. Rotenone is believed to bind covalently to the quinone-binding

site of mitochondrial C-I, although, the exact binding site within C-I is not established yet (Friedrich et al. 1994, Prieur et al. 2001). Therefore, it is unclear if the subunit of C-I, inhibited by rotenone is same as the one encoded by genes that are associated with LHON. This is of specific importance since not all mutations in C-I are associated with LHON but can also be responsible for disorders with pathologies distinctly different to LHON. For example, the mtDNA 3243G>A mutation, that causes MELAS affects the translation of mitochondrially encoded proteins (Testai and Gorelick 2010). The impairment of C-I activity forces MELAS cybrid cells, *in vitro,* to rely on anaerobic glycolysis to compensate for the ATP demand, leading to the accumulation of lactate and lactic acidosis, that is the hallmark of MELAS (Davidson et al. 2009, Kaufmann et al. 2004). Since complex I holds majority of the subunits encoded by mtDNA, it is more likely to be impaired in its function, while the compensatory activities of other complexes (C-III, IV and V) are controversially discussed (Malfatti et al. 2007, Sano et al. 1995).

Although rotenone inhibits mitochondrial C-I and elevates ROS levels, similar to the situation in LHON (Degli Esposti 1998, Fato et al. 2009, Testa et al. 2005, Treberg and Brand 2011), rotenone also has off-target effects like any other small molecule, such as disrupting Ca^{2+} homeostasis (Freestone et al. 2009, Wang and Xu 2005) that could conceivably modify the pathology of this animal model.

It has to be noted that at present there is no perfect pre-clinical model of LHON that would replicate all patient characteristics such as incomplete penetrance, gender bias, need for additional factors as well as reproducing the molecular pathology of the disease. Since the rotenone-injection model used in the present study mimics the major pathological features of LHON, such as loss of RGCs, reduced retinal thickness and loss of visual acuity and was used previously to test the efficacy idebenone (Heitz et al. 2012), this model was

selected for the current study. It has to be acknowledged that another possible limitation of this animal model is the procedure of intraocular injection of rotenone. Since the injection was performed manually, unintentional errors could be introduced by the investigator, such as leakage of rotenone out of the vitreous after injection, minor alterations of rotenone dosing (given that only one microliter is injected) or inaccurate angle of injection resulting in altered site of rotenone delivery. These factors can potentially lead to inaccurate rotenone dosing. While it was attempted to avoid these problems and no obvious evidence was observed that these problems could have compromised the current study, they cannot be ruled out as modifying factors.

Although our novel SCQs did not show any obvious adverse effects over the treatment duration, any potential SCQ-induced delayed effects cannot be ruled out. I have only assessed SCQ-treatment-induced changes to body weight, food intake and behaviour. However, since our novel SCQs are naphthoquinones and structurally similar to vitamin K (VK), they may increase blood coagulation and potentially clotting since VK is integral to the blood coagulation cascade. Although no suggestions for this putative effect have been observed in the present study, this possibility cannot be excluded and warrants more in-depth studies.

It is also worth noting the growing evidence from several cancer cell lines that VK2 exhibits some anticancer activity (Wei et al. 2010, Yoshida et al. 2003), which is believed to be mediated by ROS (Duan et al. 2016). Synthetic VK analogues were developed to increase selective toxicity to cancerous cell lines (IC_{50} 0.24 – 3.83 μM) as opposed to non-cancerous cell lines (IC_{50} 11.9 – 17.1 μM) (Prati et al. 2015). However, only 11 % of the SCQs screened for functional characterisation (9 out of the tested 83) were toxic to cells at 10 μM (Figure 19). This effect is in line with the cytoprotective role of VK2, that was

reported to prevent oxidative stress-induced cell death *in vitro* in cultured oligodendrocytes and neurons by decreasing ROS production (Li et al. 2003, Sakaue et al. 2011). Synthetic naphthoquinone derivatives were also shown to be protective against *in vitro* oxidative stress in a neuronal cell line (Josey et al. 2013), increased the *in vitro* mitochondrial ATP turn-over and overall respiration in a mouse neuronal cell line and reduced seizure activity in both zebrafish and mouse models (Chou et al. 2013, Rahn et al. 2014). VK is also reported to serve as an electron carrier that maintains ATP levels in fruit flies (Vos et al. 2012). These conflicting results suggest that although energy production and mitochondrial function can be a good target to develop new naphthoquinone-based therapeutics to treat mitochondrial dysfunction, the possibility of naphthoquinone-induced toxicity cannot be completely ruled out.

4.3 Future directions

The results of this study for the first time demonstrate a SAR for the cytoprotective ability of novel short-chain naphthoquinones. Importantly, detailed characterisation of the effect of novel SCQs on various cellular parameters, revealed that, in contrast to the current, possibly over-simplified assumption that rescuing ATP levels and reducing oxidative stress are central to restoring cell viability, my results suggest that SCQs likely act in a more complex fashion that could involve recently described novel signaling pathways, including the signaling proteins like p52Shc or Lin 28A. Consequently, future studies need to investigate this hypothesis in more detail to assess if and how these signaling pathways convey cytoprotective and mito-protective activities. Moreover, it will be essential to identify the mechanism of how exactly SCQs increase cellular levels of

Lin28A. In fact, expression control of Lin28A in general is a topic where limited information is reported. In line with the hypothesis that SCQs require a certain time to allow for signaling and gene expression events to take place, it will be important to assess the correlation between different pre-treatment times and cytoprotective activity. Although the data of this study suggest that our SCQs are potentially more potent than idebenone, it will be imperative to perform controlled dose dependency studies to obtain detailed information about potency and EC_{50} of the novel SCQs. The novel SCQs should also be further characterised with regards to their effects on energy generating pathways in the presence of mitochondrial dysfunction *in vitro,* to give real insights into the mechanism of action of these compounds. However, this will be largely influenced by the mode of action considerations explained above.

In this study, I showed the efficacy of the selected SCQs on restoring visual acuity of mice by 1-week pre-treatment and 10 weeks post-treatment following rotenone injection. Since our SCQs show significantly better activity when compared to idebenone, the necessity of pre-treating the mice with SCQs should be reinvestigated. This information along with the levels of signaling proteins in murine tissues will be invaluable to support the *in vitro* claims around the mechanism(s) of action of these compounds.

Since in this study the novel SCQs were administered orally, they would undergo first pass metabolism that can alter the efficacy of the drug. Therefore, using a different route of administration will likely provide valuable insights into the effect of first pass metabolism of these compounds and the effective dosage required. For example, administering the SCQs locally as eye drops in treating LHON would not only lower the dose required (and hence the costs incurred) but also avoid any metabolic effects and could potentially reduce any adverse effects as well. The effective dose required and

route of administration for the novel SCQs would provide a rationale and starting point to investigate tissue-specific pharmacokinetics such as uptake into the eye and brain. Moreover, since any therapeutics for LHON (with the exception of gene therapy) are likely to be taken for many years, developing a local dosage form has the potential to improve patient compliance and reduce the costs incurred.

Since our compounds appear to effectively protect against mitochondrial dysfunction, it appears obvious that they should be trialed in other disease-models that are associated with mitochondrial dysfunction such as diabetic retinopathy and ulcerative colitis. In this context, individual compounds could be selected based on physicochemical characteristics for use in particular indications. For example, compounds with a lower log D around 0 to 1, such as the acids among our SCQs, could be used orally to treat conditions such as colitis that do not require absorption into the systemic circulation. This approach would in fact even be beneficial to reduce adverse systemic effects. In contrast, cytoprotective compounds with a log D of more than 3 would, at least in theory, be more suited to enable penetration of the blood-brain-and the similar blood-eye-barrier and could therefore be superior with regards to the treatment of ophthalmological conditions.

Apart from testing the efficacy of novel SCQs in different pre-clinical models, thorough testing of basic safety, pharmacology, toxicology and drug-drug interactions are required to be able to progress selected compounds towards Investigational New Drug (IND) status. Currently, a detailed investigation into the intrinsic toxicity of the novel SCQs is currently addressed by another PhD student (Zikai Feng), who analyzes the safety, tolerability, pharmacodynamics and pharmacokinetics of the novel SCQs using a range of *in vitro* and *in vivo* assays. Another PhD student (Abraham Daniel) has developed an eye

drop formulation of two selected SCQs and is testing their efficacy in a rat-model of diabetic retinopathy.

Overall, my results have provided a promising starting point for a rational development of this class of SCQ compounds towards therapeutic drug candidates in an area that is currently still characterized by an obvious lack of therapeutic options.

Appendices

Appendix 1: Structures of novel short-chain quinones (SCQs)

Table A1: Structures of slightly polar SCQs

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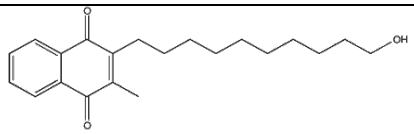
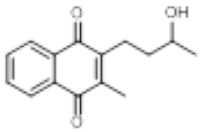
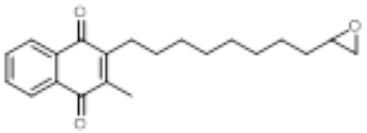
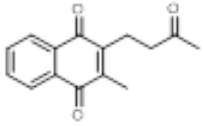
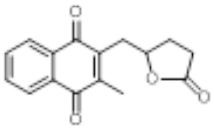
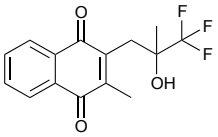
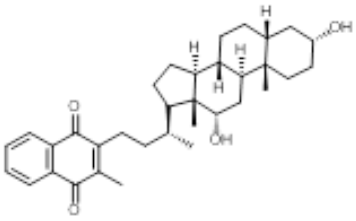
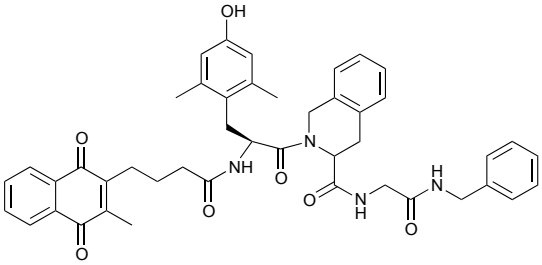
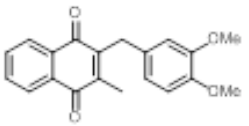
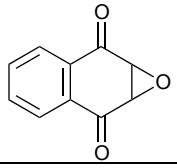
2		24	
8		25	
16		40	
18		49	
20		69	

Table A2: Structures of aliphatic SCQs

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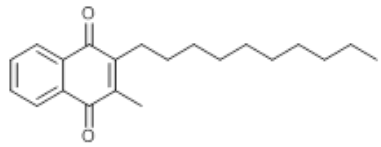
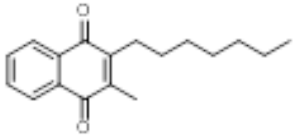
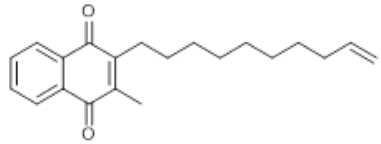
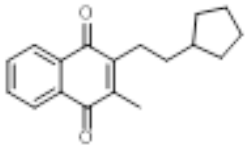
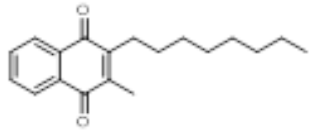
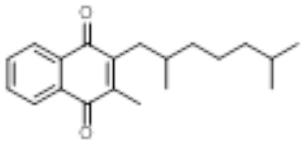
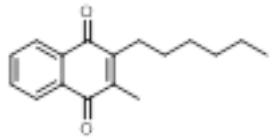
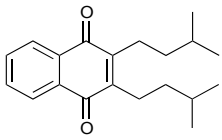
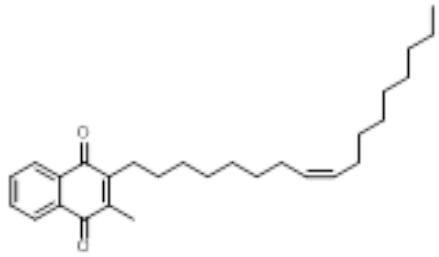
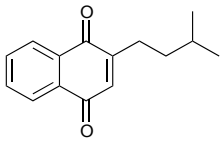
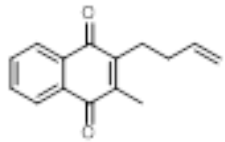
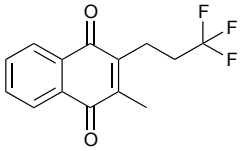
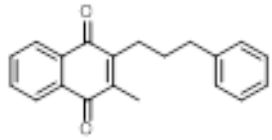
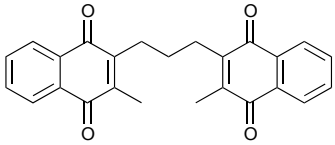
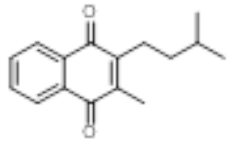
1		21	
4		22	
5		26	
6		38	
14		39	
15		41	
17		79	
19			

Table A3: Structures of acid SCQs

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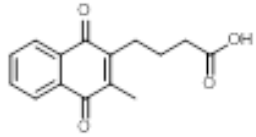
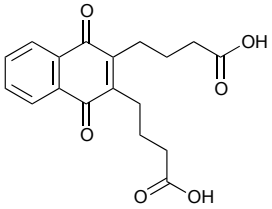
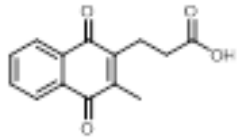
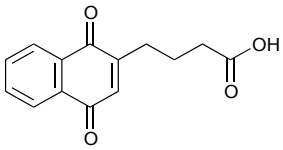
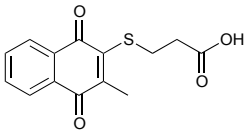
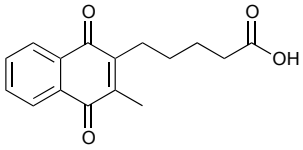
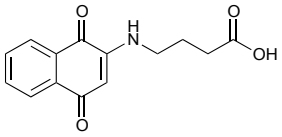
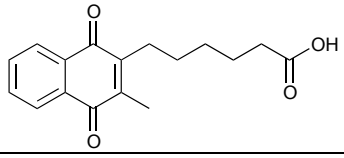
23		**58**	
28		**59**	
46		**67**	
50		**70**	

Table A4: Structures of aliphatic ester SCQs

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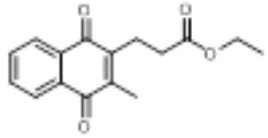
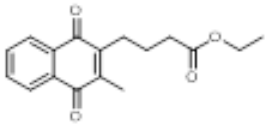
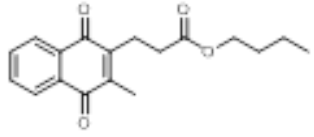
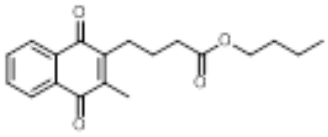
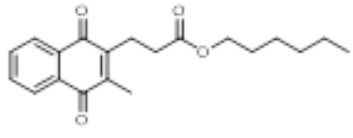
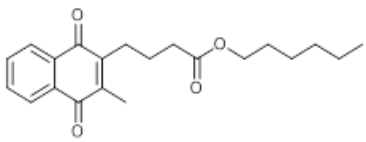
29		**32**	
30		**33**	
31		**34**	

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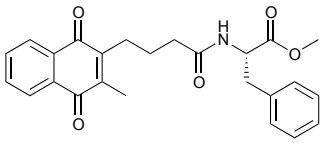
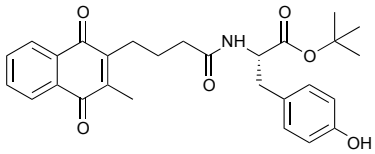
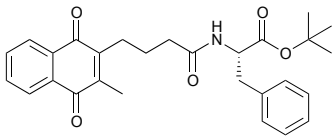
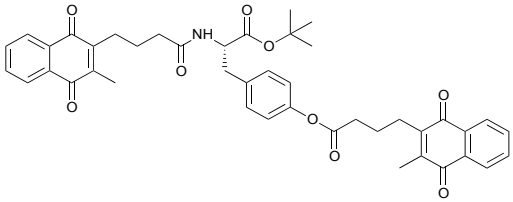
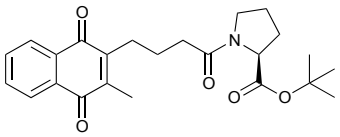
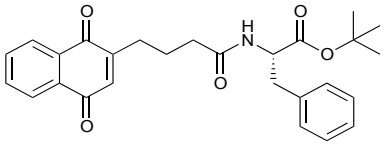
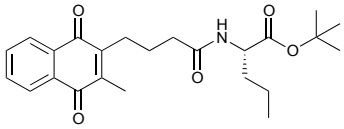
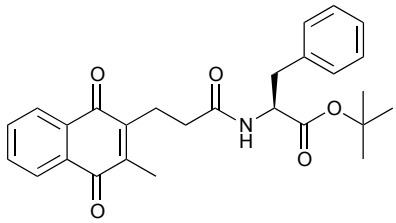
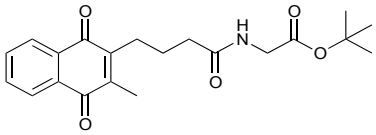
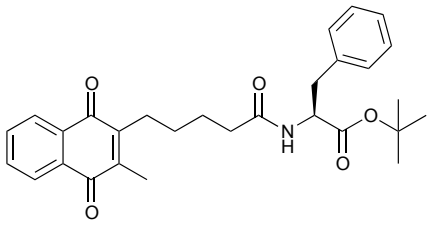
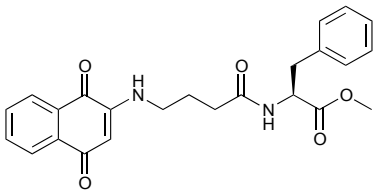
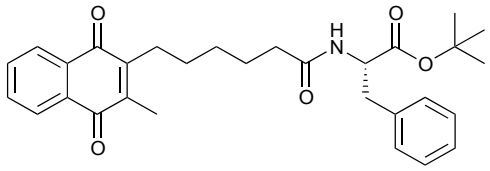
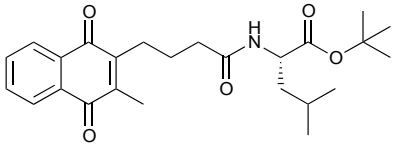
35		**55**	
36		**57**	
42		**64**	
44		**65**	
47		**71**	
51		**75**	
53			

Table A6: Structures of amino acid SCQs

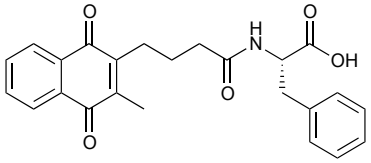
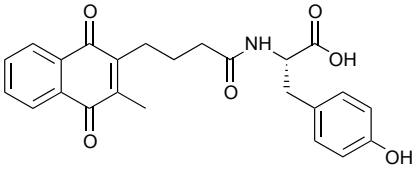
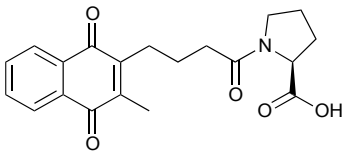
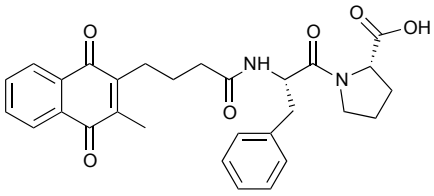
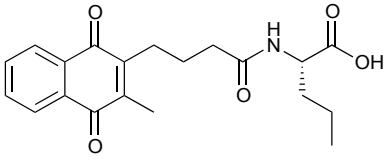
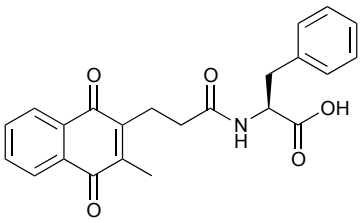
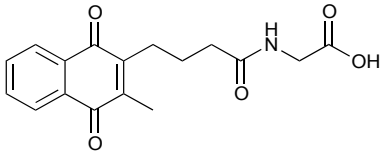
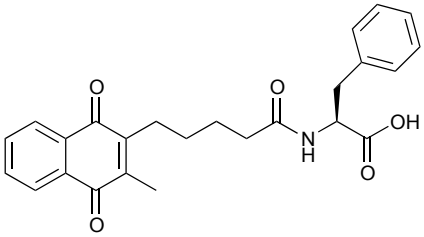
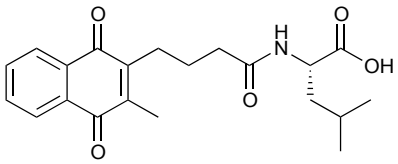
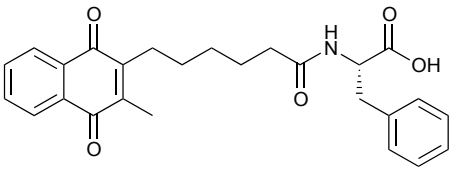
UTA#	Structure	UTA#	Structure
37		**56**	
43		**60**	
45		**66**	
48		**72**	
54		**76**	

Table A7: Structures of amino alcohol SCQs

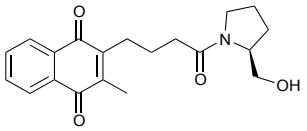
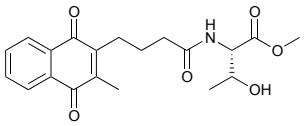
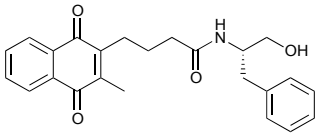
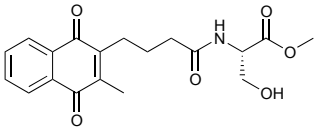
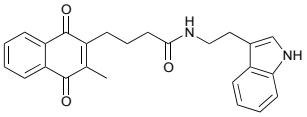
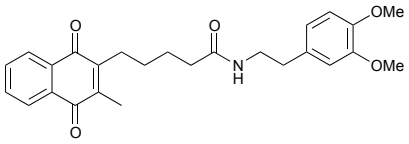
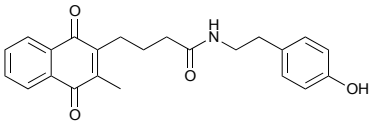
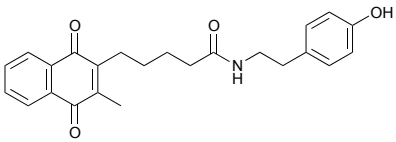
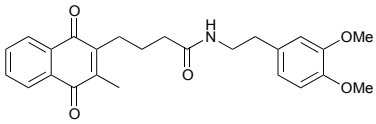
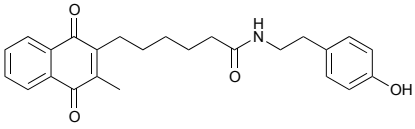
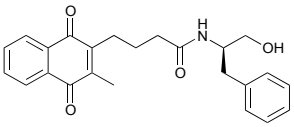
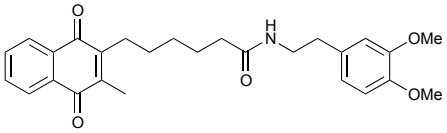
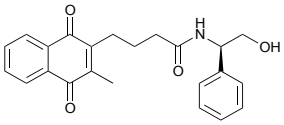
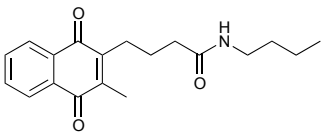
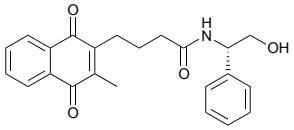
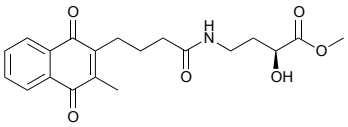
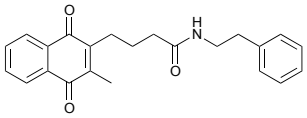
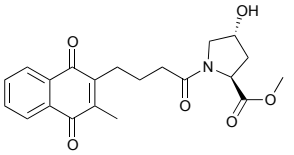
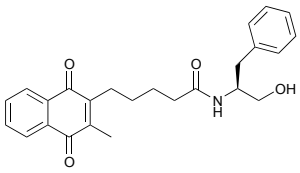
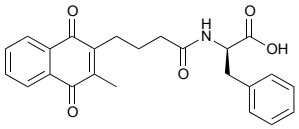
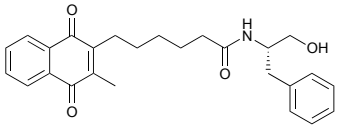
UTA#	Structure	UTA#	Structure
61		**85**	
62		**86**	
73		**87**	
74		**88**	
77		**89**	
78		**90**	
80		**91**	
81		**92**	
82		**93**	
83		**94**	
84			

Table A8: Structures of SCQs with different quinone cores

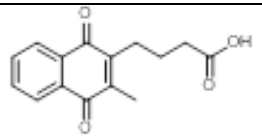
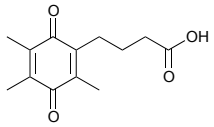
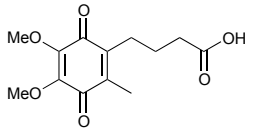
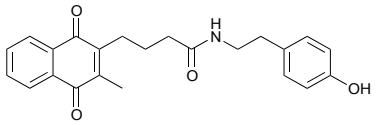
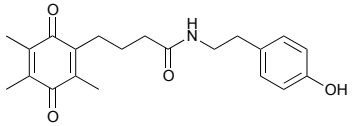
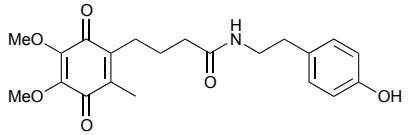
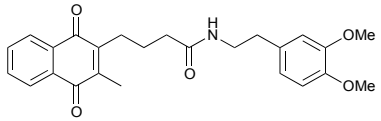
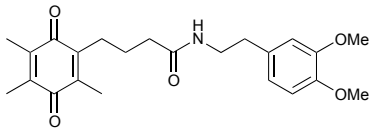
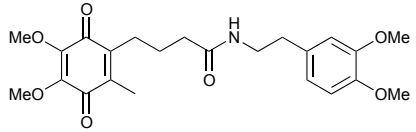
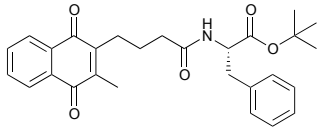
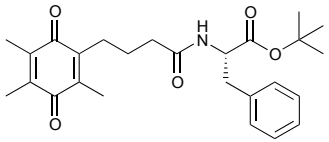
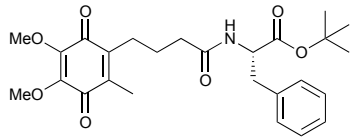
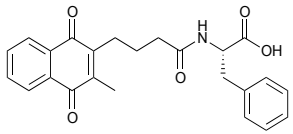
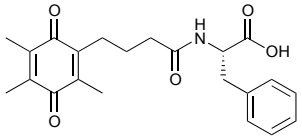
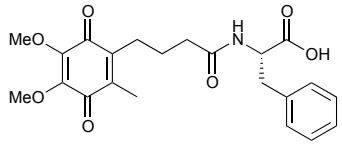
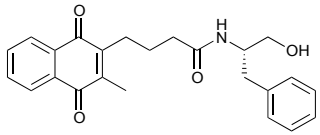
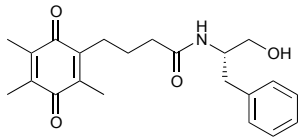
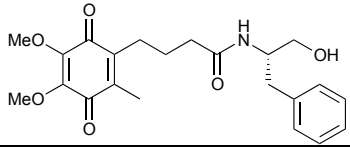
Naphthoquinone		Plastoquinone		Ubiquinone	
UTA#	**Structure**	**UTA#**	**Structure**	**UTA#**	**Structure**
23		**120**		**126**	
74		**121**		**128**	
77		**122**		**127**	
36		**123**		**130**	
37		**124**		**131**	
62		**125**		**129**	

Table A9: Structures of SCQs with different methyl group on quinone core substituted

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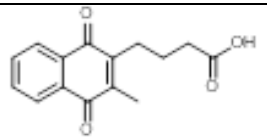
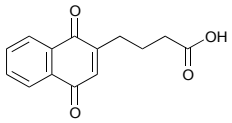
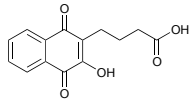
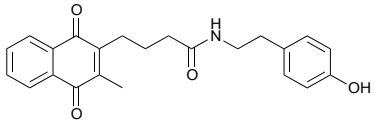
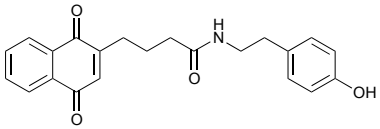
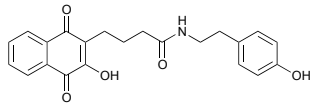
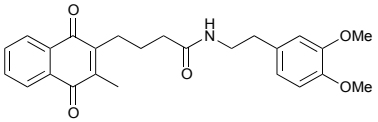
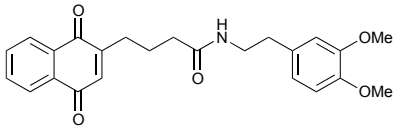
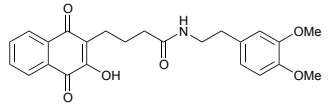
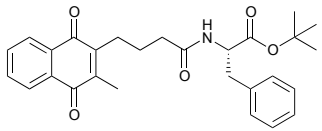
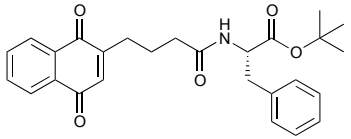
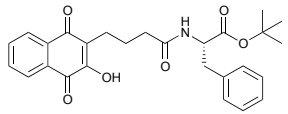
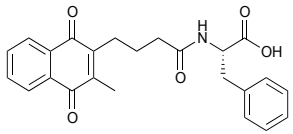
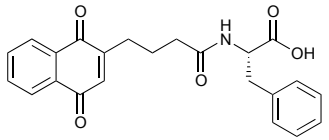
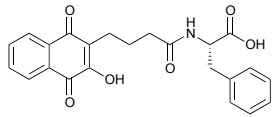
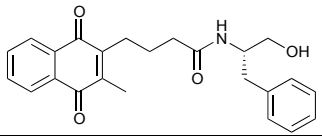
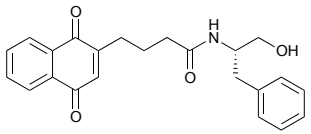
UTA#	**Structure**	**UTA#**	**Structure**	**UTA#**	**Structure**
23		**59**		**140**	
74		**113**		**143**	
77		**114**		**138**	
36		**116**		**141**	
37		**117**		**142**	
62		**115**			

Appendix 2: Summary of *in vitro* characterisation

Table A10: Summary of *in vitro* characterisation of slightly polar compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Idebenone	3.57	3.57	66.19	11.98	80.97	10.07	0.13	0.05	0.75	0.06	75.37	20.72	1.61	0.83
2	4.85	4.85	68.90	12.40	6.44	1.52	0.07	0.01	1.05	0.12	71.99	6.39	2.01	0.78
8	4.94	4.94	52.47	7.32	19.84	4.79	0.01	0.07	0.92	0.06	68.79	18.64	1.26	0.55
16	2.02	2.02	48.24	5.87	54.49	14.45	0.41	0.08	0.94	0.04	82.57	1.54	2.81	0.88
18	6.04	6.04	15.23	2.62	3.51	1.56	0.01	0.00	0.89	0.09	114.73	7.43	5.37	1.44
20	3.55	3.55	39.57	3.77	44.46	5.58	0.26	0.05	1.03	0.12	78.40	3.44	3.19	0.35
24	2.15	2.15	72.80	12.74	47.44	14.22	0.13	0.02	0.95	0.17	90.47	5.08	2.15	1.17
25	2.18	2.18	56.68	6.75	34.21	6.16	0.15	0.02	0.92	0.05	97.40	9.45	1.55	0.37
40	2.59	1.51	21.33	10.19	79.87	5.83	0.64	0.15	0.92	0.07	65.30	6.61	2.16	1.02
49	5.27	5.27	23.80	4.77	89.30	16.43	0.11	0.03	N/A		148.96	20.77	1.94	0.25
69	0.87	0.87	3.34	0.32	80.12	13.29	0.01	0.00	0.97	0.06	52.78	3.29	3.10	0.77
Mean	**3.46**	**3.36**	**42.59**		**49.15**		**0.17**		**0.93**		**86.07**		**2.47**	**0.76**
SD	**1.64**	**1.73**	**23.65**		**30.94**		**0.19**		**0.08**		**26.73**		**1.15**	**0.37**

Table A11: Summary of *in vitro* characterisation of aliphatic compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	6.29	6.29	5.57	1.00	1.97	0.49	-0.01	0.00	0.97	0.04	63.84	4.00	1.38	0.89
4	5.98	5.98	34.11	2.21	3.41	0.84	0.07	0.01	0.89	0.04	106.20	2.47	1.92	0.74
5	5.40	5.40	50.99	5.79	6.64	1.52	0.02	0.01	1.06	0.08	67.37	5.72	2.33	0.77
6	4.51	4.51	42.89	5.46	14.51	9.64	0.05	0.00	N/A		57.79	1.98		
14	9.04	9.04	6.19	0.64	2.11	0.38	0.01	0.00	0.94	0.02	56.35	9.99	1.54	0.74
15	3.32	3.32	52.15	10.42	22.37	4.55	0.16	0.04	0.92	0.01	67.49	3.78	2.09	0.20
17	4.75	4.75	18.25	1.18	16.58	1.91	0.05	0.01	0.94	0.04	73.08	11.45	2.85	0.43
19	3.91	3.91	24.90	1.50	4.15	0.85	0.03	0.01	0.87	0.05	65.19	2.00	2.08	0.26
21	4.95	4.95	33.88	5.62	19.60	6.11	0.02	0.01	0.95	0.13	75.49	0.99	1.44	0.51
22	4.33	4.33	25.68	3.36	32.99	11.15	0.06	0.01	N/A		72.82	2.05	1.08	0.95
26	5.53	5.53	56.77	6.04	11.69	0.73	-0.01	0.01	0.86	0.03	154.41	2.40	2.52	0.78
38	5.53	5.53	63.93	3.47	32.70	6.06	0.00	0.00	1.05	0.02	75.14	6.10		
39	3.51	3.51	65.93	8.35	79.30	8.41	0.11	0.03	0.94	0.03	67.35	9.18	1.84	1.03
41	3.36	3.36	51.70	3.71	73.50	4.75	0.17	0.02	0.94	0.03	51.16	3.55	1.47	0.41
79	4.55	4.55	11.85	1.11	78.16	7.27	0.05	0.01	1.00	0.04	138.00	16.53	18.38	6.06
Mean	**5.00**	**5.00**	**36.32**		**26.65**		**0.05**		**0.95**		**79.45**		**3.15**	**1.06**
SD	**1.45**	**1.45**	**20.42**		**27.90**		**0.06**		**0.06**		**29.96**		**4.60**	**1.53**

Table A12: Summary of *in vitro* characterisation of acid compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
23	2.30	-0.93	21.30	2.98	19.13	1.68	-0.02	0.01	0.91	0.02	113.02	1.31	2.67	0.70
28	1.85	-1.40	32.03	3.34	13.29	1.29	0.02	0.01	0.94	0.11	122.87	2.95	1.82	0.27
46	1.88	-1.43	80.50	11.47	53.70	7.13	0.06	0.01	0.94	0.03	56.31	3.10	1.23	0.63
50	0.77	-2.53	47.00	10.03	27.30	6.92	0.00	0.00	1.00	0.08	74.72	1.51	2.10	0.58
58	2.31	-4.03	32.28	10.77	31.32	7.96	-0.01	0.00	0.97	0.05	56.28	1.30	2.20	0.25
59	1.90	-1.37	44.55	15.04	79.88	20.17	0.01	0.00	0.99	0.02	48.12	1.52	2.13	0.65
67	2.74	-0.49	74.89	18.46	97.09	18.97	0.08	0.02	0.96	0.04	50.08	1.22	2.81	0.11
70	3.19	0.02	66.82	5.96	91.79	20.18	0.02	0.01	0.98	0.05	54.32	2.95	1.93	0.71
Mean	**2.12**	**-1.52**	**49.92**		**51.69**		**0.03**		**0.96**		**71.96**		**2.11**	**0.49**
SD	**0.72**	**1.26**	**21.82**		**33.83**		**0.03**		**0.03**		**29.60**		**0.49**	**0.24**

Table A13: Summary of *in vitro* characterisation of aliphatic ester compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
29	2.36	2.36	10.33	2.31	27.35	7.34	0.04	0.00	0.94	0.07	113.43	16.63	2.56	0.31
30	3.32	3.32	17.74	0.63	11.77	2.72	0.02	0.01	0.98	0.04	111.28	3.06	0.86	0.35
31	4.21	4.21	16.10	1.48	8.96	2.91	0.01	0.00	0.96	0.13	107.13	2.70	1.71	0.15
32	2.80	2.80	15.69	1.22	15.37	2.00	0.04	0.01	0.88	0.14	104.42	1.20	1.51	0.56
33	3.77	3.77	15.14	1.41	9.64	1.50	0.01	0.00	N/A		102.32	3.70	2.64	0.20
34	4.66	4.66	18.91	1.06	17.79	11.55	0.01	0.00	1.01	0.08	51.89	2.62	1.66	0.18
Mean	**3.52**	**3.52**	**15.65**		**15.15**		**0.02**		**0.95**		**98.41**		**1.82**	**0.29**
SD	**0.87**	**0.87**	**2.96**		**6.87**		**0.02**		**0.05**		**23.16**		**0.68**	**0.15**

Table A14: Summary of *in vitro* characterisation of amino ester compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
35	3.56	3.56	76.20	17.28	76.67	7.58	0.05	0.01	0.96	0.03	56.88	5.09	1.96	0.41
36	4.62	4.62	54.50	12.06	81.03	9.41	0.18	0.03	0.84	0.09	56.46	3.11	2.97	0.53
42	3.23	3.23	64.68	6.54	79.07	9.32	0.23	0.04	0.96	0.05	46.96	1.07	1.86	0.13
44	3.93	3.39	60.36	7.41	80.47	14.03	0.28	0.05	0.95	0.04	47.18	1.32	2.50	0.38
47	2.39	2.39	74.40	5.64	76.40	5.24	0.42	0.11	0.90	0.01	75.94	6.59	1.69	0.64
51	2.04	2.04	55.30	13.81	46.50	8.01	0.00	0.00	0.98	0.09	69.16	12.95	1.74	0.61
53	4.22	4.22	57.17	12.05	87.60	5.02	0.20	0.05	0.97	0.06	92.51	6.42	2.10	0.10
55	4.31	4.31	78.60	14.13	94.76	4.14	0.11	0.02	0.94	0.07	75.48	3.79	1.02	0.48
57	6.75	6.75	59.97	26.86	79.18	9.43	0.03	0.01	N/A		53.66	1.39	1.60	0.26
64	4.22	4.22	69.40	14.49	84.41	16.90	0.14	0.02	0.97	0.07	61.31	3.92	1.71	0.10
65	4.17	4.17	60.08	13.52	91.07	17.51	0.14	0.03	0.89	0.04	76.58	1.95	1.34	0.46
71	5.06	5.06	70.53	19.51	92.22	10.29	0.07	0.01	0.87	0.06	56.47	12.41	5.14	0.15
75	5.51	5.51	61.35	7.27	95.42	18.61	0.07	0.02	0.95	0.06	53.57	1.66	1.77	1.16
Mean	**4.15**	**4.11**	**64.81**		**81.91**		**0.15**		**0.93**		**63.24**		**2.11**	**0.42**
SD	**1.24**	**1.26**	**8.16**		**12.60**		**0.12**		**0.05**		**13.62**		**1.03**	**0.29**

Table A15: Summary of *in vitro* characterisation of amino acid compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
37	3.42	0.12	100.30	17.34	64.20	3.95	0.03	0.01	0.90	0.04	50.31	4.80	1.72	0.43
43	2.04	-1.32	92.70	7.59	46.57	5.67	0.03	0.01	0.98	0.03	50.50	3.46	1.93	0.57
45	2.73	-0.58	61.00	19.11	45.90	8.31	0.02	0.01	0.94	0.03	44.40	1.27	1.96	0.34
48	1.19	-2.18	53.63	22.46	56.60	13.49	0.06	0.01	0.91	0.06	64.02	4.66	2.27	0.33
54	3.02	-0.26	98.70	10.87	66.10	12.64	0.01	0.01	0.97	0.08	64.75	3.04	1.84	0.28
56	3.12	-0.26	29.73	12.23	54.49	8.36	0.01	0.00	0.96	0.05	68.25	7.98	1.72	0.22
60	3.15	-0.08	34.78	6.24	50.70	5.83	0.02	0.01	0.88	0.08	57.73	9.26	1.99	0.59
66	2.97	-0.39	66.94	3.91	73.31	23.74	0.05	0.01	0.95	0.06	48.91	1.53	1.97	0.98
72	3.86	0.74	90.75	15.62	84.80	29.49	0.05	0.03	0.89	0.13	46.77	1.19	1.74	0.15
76	4.31	1.25	68.12	14.11	51.44	6.95	0.01	0.01	0.96	0.04	57.17	3.02	2.80	0.86
Mean	**2.98**	**-0.30**	**69.66**		**59.41**		**0.03**		**0.93**		**55.28**		**1.99**	**0.47**
SD	**0.88**	**0.96**	**25.62**		**12.58**		**0.02**		**0.04**		**8.32**		**0.33**	**0.27**

Table A16: Summary of *in vitro* characterisation of amino alcohol compounds

UTA#	LogP	LogD	Viability (% Control)		ATP levels (% Control)		Reduction (ΔA_{450} nm)		Basal lipid peroxidation (FU)		Lactate levels (µmol/mg)		Beta-hydroxy butyrate levels (µmol/mg)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
61	1.71	1.71	100.70	28.37	78.57	5.12	0.23	0.07	0.93	0.03	56.42	2.18	2.15	0.72
62	3.10	3.10	93.08	13.67	91.22	7.23	0.22	0.03	0.90	0.07	67.05	2.17	2.40	0.29
73	3.83	3.83	86.22	9.41	96.07	8.30	0.12	0.03	0.96	0.04	51.85	3.62	1.04	0.48
74	3.43	3.43	91.73	15.63	100.67	16.01	0.20	0.06	0.87	0.07	50.91	1.72	1.69	0.38
77	3.41	3.41	95.92	19.39	99.12	19.16	0.21	0.06	0.76	0.11	46.33	1.39	2.60	0.40
78	3.10	3.10	79.95	21.09	84.23	9.15	0.13	0.04	0.96	0.05	44.66	0.91	2.04	0.27
80	2.81	2.81	87.57	19.67	89.06	10.87	0.22	0.04	0.94	0.07	63.23	8.98	1.96	0.36
81	2.81	2.81	83.77	19.93	89.90	11.74	0.25	0.07	0.88	0.05	81.79	11.95	1.42	0.60
82	3.73	3.73	64.12	2.88	84.29	6.47	0.44	0.04	0.80	0.08	77.84	1.03	1.78	0.44
83	3.54	3.53	60.30	7.55	73.45	15.95	0.43	0.05	0.95	0.03	101.77	4.42	3.78	1.50
84	3.99	3.99	74.36	3.77	90.41	6.06	0.34	0.02	0.93	0.12	40.19	2.82	1.86	0.26
85	1.28	1.28	61.42	9.62	79.28	3.18	0.17	0.02	0.91	0.08	45.96	2.04	2.14	0.35
86	0.86	0.86	49.50	10.68	78.40	11.02	0.04	0.01	0.98	0.05	48.43	3.09	1.28	0.23
87	3.86	3.86	63.55	14.70	79.40	19.91	0.34	0.06	0.97	0.02	50.44	1.41	2.53	0.42
88	3.87	3.87	91.81	9.85	80.17	15.58	0.18	0.03	0.90	0.06	42.92	4.34	2.18	0.71
89	4.31	4.31	85.17	9.46	89.96	11.51	0.15	0.04	0.93	0.06	37.92	1.31	1.03	0.17
90	4.30	4.30	68.03	8.02	87.59	13.13	0.18	0.06	0.93	0.04	45.50	3.18	1.79	0.67
91	3.04	3.04	82.01	7.11	77.44	8.83	0.17	0.04	0.93	0.05	73.75	3.83	1.66	0.62
92	0.92	0.92	40.47	3.63	72.43	10.41	N/A		0.96	0.02	102.15	3.19	1.80	0.39
93	1.03	1.03	74.38	4.11	82.67	8.12	N/A		0.93	0.03	67.23	5.09	1.42	0.14
94	3.42	0.12	71.70	7.39	65.32	3.80	N/A		0.85	0.18	59.68	3.49	1.65	0.18
Mean	**2.97**	**2.81**	**76.47**		**84.27**		**0.22**		**0.91**		**59.81**		**1.91**	**0.46**
SD	**1.13**	**1.28**	**15.86**		**8.97**		**0.10**		**0.06**		**18.71**		**0.61**	**0.30**

Appendix 3: Animal Ethics approval

UNIVERSITY of TASMANIA

Animal Ethics Committee
ETHICS APPROVAL PERMIT

Office of Research Services
Phone : 03 62267283
Fax: 03 62267148
animal.ethics@utas.edu.au

To: Dr Anna King

From: Christy Nixon

Date: 02 November 2016

Project: A0016080 - Novel naphthoquinones against neurodegeneration

Approved on: 31 October 2016

Approval expires: 31 October 2019

1st Annual Report due: 31 October 2017

Please read this permit carefully as approval may be withdrawn for non-compliance with the conditions stated below.

The Animal Ethics Committee has approved the above project and a copy of the initial application document is attached. The approval is subject to the review and AEC approval of an annual report which is due before the approval anniversary. **Please note the due date in your diary.**

As the Responsible Investigator, you MUST ensure that:

1. All aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

2. The project is conducted in accordance with the provisions of the Tasmanian Veterinary Surgeons Act 1987 and Veterinary Surgeons Regulations 2012. If the project involves a veterinary service or other animal service, it is **your responsibility** to contact the University Veterinarian to discuss the legal requirements of competency assessment.

3. The University Veterinarian and the Animal Ethics Committee are promptly notified of any unexpected event which was not considered in

the initial application and impacts on the welfare of any animal directly or indirectly involved in the project.

4. You contact the University Veterinarian to advise when and where your experiments will be conducted. Sufficient notice needs to be given so that an inspection can be easily arranged.

5. In the event of any unexpected death, you contact the University Veterinarian to perform an autopsy.

6. A full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.

7. That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.

The project is approved for a maximum of 3 years. If the project is to continue past the expiry date, a new initial application will need to be submitted.

If the investigation necessitates a Parks & Wildlife permit or other permits, you are required to send copies to animal.ethics@utas.edu.au before commencing work.

Kind regards,

Christy

Christy Nixon
Research Operations Administration Officer
Office of Research Services
University of Tasmania
Level 3, 301 Sandy Bay Rd
Hobart TAS 7000
T: +61 3 6226 7592 | E: Christy.Nixon@utas.edu.au
www.utas.edu.au/research

UNIVERSITY of TASMANIA

Appendix 4: Summary of mice information

Table A17: Mice from sham-treated group

Batch	Mice code	Parents	Age (d)	Body weight (g)
B1	4596	3787*4036	77	25.01
B1	4597	3787*4036	77	23.99
B1	4622	4233*4021	74	22.70
B2	4662	4123*2796	63	24.30
B3	4935	3984*3909	79	27.39
B4	4967	4690*4686	67	22.06
B4	4975	4692*4688	66	22.30
B5	4985	4693*4689	67	21.88
B5	4992	3787*4036	66	21.77
B5	4998	4691*4687	65	23.50
		Mean	**70.1**	**23.5**
		SD	**6.0**	**1.8**

Table A18: Mice from idebenone-treated group

Batch	Mice code	Parents	Age (d)	Body weight (g)
B1	4624	4233*4021	74	26.40
B1	4625	4233*4021	74	22.57
B1	4626	4233*4021	74	23.12
B2	4674	4122*4013	61	25.88
B3	4937	3984*3909	79	26.49
B4	4968	4690*4686	67	23.26
B4	4977	4692*4688	66	22.13
B4	4982	4693*4689	64	21.48
B5	4991	3787*4036	66	22.01
B5	4999	4691*4687	65	25.05
		Mean	**69.0**	**23.8**
		SD	**5.8**	**1.9**

Table A19: Mice from #37-treated group

Batch	Mice code	Parents	Age (d)	Body weight (g)
B2	4659	4123*2796	63	24.27
B2	4660	4123*2796	63	25.32
B2	4668	3787*4036	63	22.73
B3	4928	4227*4362	79	24.29
B3	4936	3984*3909	79	22.79
B4	4978	4692*4688	66	22.93
B4	4983	4693*4689	64	22.98
B5	4986	4693*4689	64	21.19
B5	4990	3787*4036	66	22.59
B5	5000	4691*4687	65	23.55
		Mean	**67.2**	**23.9**
		SD	**6.3**	**1.0**

Table A20: Mice from #77-treated group

Batch	Mice code	Parents	Age (d)	Body weight (g)
B2	4661	4123*2796	63	24.63
B2	4669	3787*4036	63	23.61
B2	4673	4122*4013	61	25.10
B3	4929	4227*4362	79	23.51
B3	4952	3990*3915	74	25.17
B4	4949	3990*3915	80	25.24
B4	4976	4692*4688	66	23.38
B4	4984	4693*4689	64	23.90
B5	4987	4693*4689	67	22.58
B5	4989	3787*4036	66	22.66
B5	5006	3991*3914	63	23.38
		Average	**67.8**	**23.9**
		SD	**6.7**	**1.0**

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